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Suggested short title (54 letters):

I. ADRENAL CORTEX IN STRESS

II. NATURAL GLYCOGENOLYTIC SUBSTANCES

I. INVESTIGATION OF SOME FACTORS AFFECTING THE PITUITARY-ADRENAL SYSTEM.

II. STUDIES ON THE GLYCOGENOLYTIC HYPERGLYCEMIC HORMONE OF THE PANCREAS.

THESIS

BY

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

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Montreal,
August, 1953.

ACKNOWLEDGEMENTS

The author gratefully thanks the following for their assistance:-

Dr. R.D.H. Heard, Dr. K. Savard and

Dr. M.M. Hoffman, Dr. H. Selye,

Dr. J.L. Prado,

for suggesting the problems studied and for directing the Research.

Dr. R.D. Stewart, for invaluable assistance in the preparation of the manuscript.

Dr. G. Masson, Dr. M. Chaput, Dr. D. MacFarlane, Miss Joan Dewar, Miss Lyse Farley and Miss Thérèse Hansen for help with specific experiments.

Drs. R.E. Scott and A.M. Fisher, Connaught Laboratories, Toronto, for their generous supply of pancreatic extract.

Miss Elizabeth Solheim for her care in typing this manuscript.

Mrs. W.A. Keddy for preparing the graphic material.

The Dazian Research Foundation and the Banting Research Foundation for financial aid.

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Part I

INVESTIGATION OF SOME FACTORS AFFECTING THE PITUITARY-ADRENAL SYSTEM

A. Introduction

The purpose of this study was to investigate the mechanism of secretion of the pituitary-adrenal system by studying some factors which modify the response of these glands to stress. Two such factors were considered: (a) exogenous cortical hormone (represented by desoxycorticosterone) and (b) dietary protein. Both these agents had been previously studied by other investigators, but it was thought of interest to investigate them further because some of the results reported were contradictory. Furthermore, the possibility that these glands may play an important rôle in the pathogenesis of the so-called diseases of adaptation, made it of interest to know how their secretory activity can be modified by external agents.

With the introduction of the concept of the General Adaptation Syndrome by Selye (1,2,3) a rather large mass of independent observations was unified within the confines of a single theory. Briefly this concept postulates that when subjected to stress, an animal, besides undergoing adaptive changes specific to the particular stress, also undertakes a general non-specific defense which is mediated by the pituitary-adrenal system. The pituitary-adrenal reaction is believed to be a defense or resistance mechanism common to all types of stress and to consist of four phases: shock, countershock, resistance and exhaustion, terminating in death should the stress be sufficiently severe. Either adrenalectomy or hypophysectomy affects only the resistance stage by considerably shortening it, but does not influence the shock and countershock phases (4).

As early as 1940 Selye and his collaborators concluded that the resistance to stress of normal animals could be increased by cortical

extracts (5) and by corticosterone, a carbohydrate-active hormone of the adrenal cortex (6,7). These authors concluded that in general the gluco-corticoids (having an oxygen atom at "carbon eleven" of the steroid nucleus) were more active in raising the resistance of non-adrenalectomized animals above normal values than the mineralo-corticoids (which have no oxygen atom at "carbon eleven"). These conclusions have not gone unchallenged, and many attempts to prove that cortical extracts or purified corticoids increase the resistance of the intact animal to stress have failed (8-15); nevertheless, repeatedly reports have appeared in the literature demonstrating the beneficial effect of cortical hormones against the damaging action of stressful agents (16-19).

Representative of this controversial situation are Ingle's experiments (20) demonstrating the inability of adrenocortical extract to improve the work performance or to prevent hypoglycemic coma in forcibly exercised normal rats. Similarly the same author found that large doses of adrenocortical extract did not increase the survival time of rats subjected to scalding (10); whereas the same treatment increased the incidence of survival of normal rats after intraperitoneal administration of peptone solution (21).

Despite these inconsistent results, it is generally admitted that the adrenalectomized animal has a decreased resistance to damaging stimuli (22,23), and therefore it may be concluded that cortical secretion contributes to the animal's defense in stress; although it is not quite clear whether or not this is the factor limiting the ability of the normal organism to withstand severe stress.

Since under stress the adrenal cortex becomes hyperactive (23, 24,25) it appeared of interest to investigate what factors can modify the gland's secretory activity, in an effort to elucidate the mechanisms that regulate the secretion of cortical hormones. Two such factors have been studied: one hormonal and one dietary.

B. Hormonal Studies

1. Review of the Literature

The regulation of the rate of secretion of the hypophyseo-adrenal system is not as yet fully understood, and several hypotheses have been advanced in an effort to explain the pertinent experimental observations.

It has been established that the adrenal cortex increases its secretory activity under the influence of stress (23,24,25), and that in the absence of the pituitary, stress does not bring about the morphological and biochemical changes that characterize its action on the adrenal cortex (25,26,27). The pituitary, however, is not essential to maintain a minimal secretion of cortical hormone, as shown by the inability of adrenalectomized animals to survive the operation for more than a few days (28,29); whereas after hypophysectomy, the animal may survive for long periods of time (30,31). Furthermore the administration of adrenocortical extract increases the resistance of the hypophysectomized animal to stress (32,33). This much, at least, is common to the different controversial theories which attempt to explain how stress results in cortical hyperactivity.

An interpretation that has received considerable experimental support in recent times is the "peripheral-humoral concept" of Sayers (23). Briefly, it suggests that adrenocorticotrophin secretion by the pituitary varies inversely with the concentration of circulating cortical hormone. This hypothesis is based on the reasonable assumption that under conditions of stress there is an increased peripheral utilization of cortical hormone, which results in a temporary decrease of circulating cortical hormone. This drop in concentration stimulates the hypophysis to discharge adrenocorticotrophin and thus brings the blood titer of corticoids back towards its initial level.

The experimental evidence supporting the peripheral-humoral theory can be divided into two main types: (a) chronic experiments in which the morphological and histological changes that the adrenal cortex undergoes after stimulation are inhibited by the administration of cortical substances, and (b) acute experiments in which the biochemical changes that stress induces in the cortical tissue are partially or totally prevented by the administration of cortical substances.

To the first class belong the experiments of Ingle and his co-workers (34) who showed that the administration of adreno-cortical extract (ACE) resulted in adrenal atrophy; a condition that could be reversed by the simultaneous administration of pituitary extracts. ACE also prevents the adrenal hypertrophy caused by thyroxine treatment (35). Desoxycorticosterone acetate (DCA) has been shown to cause adrenal involution in the normal animal (36,37) and to inhibit the adrenal hypertrophy that follows exposure to non-specific stressing agents such as: exercise (38,39) oestrogen overdosage (40), and

electroshock convulsions (41).

The first quantitative studies in the biochemical composition of the stimulated adrenal cortex were reported by Sayer's group in 1942 (42). These authors showed that the administration of adrenocorticotrophin (ACTH) to rats resulted in a decrease of both the ascorbic acid and the cholesterol concentrations of the adrenals. Stress was shown to induce similar biochemical changes in normal rats. (43).

The administration of ACE prevents the decrease in adrenal ascorbic acid that follows exposure to cold, but fails to do so after administration of ACTH (44). Sayer's interpretation of this observation is that the cortical extract inhibits ACTH release by the pituitary, and that it does not directly interfere with cortical activity; since exogenous ACTH can stimulate the adrenal cortex in the presence of a high corticoid titer in the blood. The following crystalline cortical steroids have also been shown to inhibit the adrenal-ascorbic-acid response to stress: hydroxy-corticosterone, 17-hydroxy-11-dehydrocorticosterone, corticosterone, and desoxy-corticosterone. (43). Presumably these cortical substances act by maintaining a high enough concentration of hormone in the blood to prevent an increase in the secretion of ACTH.

Further evidence is offered by Long's experiments showing that ACE prevents the depletion of adrenal ascorbic acid following exposure to cold or after unilateral adrenalectomy (45), or following the administration of epinephrine (46).

A roughly quantitative relationship has been shown to exist

between the stress applied and the dose of corticoid required to prevent adrenal ascorbic acid depletion (41,47), and Sayers concludes that the extent to which circulating corticoids, whether exogenous or not, can block the release of ACTH by the anterior pituitary, is a function of the rate at which the periphery utilizes cortical hormone.

A corollary of the peripheral-humoral concept is that by its self-regulatory nature the corticoid titer of the blood is maintained within physiological limits, provided the pituitary-adrenal axis can cope with the variations in peripheral demand for adrenocortical secretion.

If this is the only mechanism that regulates the rate of secretion of the pituitary-adrenal system, it should be impossible to arrive at a condition in which the organism would be exposed to an abnormally high concentration of circulating corticoids for prolonged periods of time. This very condition, however, was the basis of the interpretation of the pathogenesis of the diseases of adaptation held by Selye about 1947 when the present work was done. The cordiovascular, renal and joint lesions which Selye has grouped and defined as "diseases of adaptation" are, in his opinion, the result of endogenous hormonal overdosage (4).

The concept of the diseases of adaptation was arrived at from the similarity of the pathological changes observed in experimental animals after hormonal overdosage or exposure to stress and conditions found in human pathology. Selye and his coworkers have shown that DCA overdosage

(particularly in sensitized animals) produces cardio-vascular lesions (48,49,50,51), hypertension (49,50),arthritic joint lesions (49,52), and nephrosclerosis (48,50,53,54), all of which find their counterpart in human pathology.

Selye has further shown that similar renal, cario-vascular and joint lesions could be elicited in sensitized rats by chronic treatment with a crude anterior pituitary preparation (55,56) which also causes hypertension (57). These effects of anterior pituitary preparations are either mediated by the adrenal cortex or require the presence of cortical hormone, since they do not produce renal or cardio-vascular damage in the adrenalectomized animal (58). Seyle suggested that anterior lobe extracts act through their action on the adrenal cortex, causing it to release an excessive amount of salt-active corticoids which in turn would reproduce the symptoms elicited by DCA overdosage (4).

These observations pointed to the possibility that the diseases of man which could be experimentally reproduced by overdosage with DCA or anterior pituitary preparations, could be the result of an excessive production of endogenous mineralo-corticoids. Thus these diseases would result from the efforts made by the human organism to adapt to intense and prolonged stressful situations. Support of this view is afforded by experiments showing that such stressing agents as exposure to cold, formaldehyde injections and exercise, elicit hypertension and nephrosclerosis in sensitized animals (59). The blood chloride changes in both normal (60) and adrenalectomized animals (61)

subjected to stress, and the ability of diets rich in sodium chloride to exacerbate the pathological changes due to DCA overdosage (48,50,54, 62), led Selye to the conclusion that the diseases of adaptation were the result of an excessive production of endogenous mineralo-corticoids. On the other hand the changes in carbohydrate metabolism which occur during the general adaptation syndrome suggested that an increased secretion of gluco-corticoids may result as a defensive response to stress (4,60,61).

The observations reported here were made in 1947 and since then the interpretation of the pathogenesis of the diseases of adaptation has been somewhat reviewed by its originator in the light of new experimental data. The work of Hench, Kendall et al. (63,64,65) threw new light on the collagen diseases. These authors established that ACTH and particularly Kendall's compound E (17-hydroxy-11-dehydro-corticosterone, also known as cortisone) could reverse the course of rheumatoid arthritis and related diseases, producing a rapid regression of the articular and systemic manifestations. Since then numerous investigators have confirmed these results (66,67) and have added to an increasing list of maladies to which either ACTH or cortisone bring relief.

Cortisone and ACTH have been shown to reverse most of the pathological changes produced by DCA or crude anterior pituitary treatment, with the exception of renal damage, which is aggravated by gluco-corticoids (68). This action of ACTH is incompatible with the rôle Selye assigned to it in the pathogenesis of the diseases of adaptation. Furthermore there is evidence that the zona glomerulosa

of the adrenal cortex selectively secretes mineralo-corticoids (37,69) and that its secretory activity is partially regulated by the Na/K ratio of the blood (70,71) and is somewhat independent of the anterior pituitary, since hypophysectomy does not result in atrophy of this zone. (72). Stress and anterior pituitary preparations primarily stimulate the zona fasciculata which is thought to be the site of secretion of glucocorticoids (73,74), whose protective action in situations of stress has been referred to above (6,7,68). Corcoran and his collaborators have pointed out other serious objections to Selye's original claim that chronic treatment with pituiatry preparations elicit pathological lesions by increasing adrenocortical secretion of DCA-like steroids. These authors have shown that ACTH tends to decrease the blood pressure of hypertensive rats (75) and they have also pointed out some qualitative differences in the response of sensitized rats treated with either DCA or anterior lobe preparations: (a) only a small number of animals developed hypertension after pituitary treatment whereas all animals responded to DCA administration; (b) DCA produced severe diuresis while anterior pituitary treatment induced only a slight initial increase in urine formation (76).

To correlate these findings with his own observations that crude anterior pituitary preparations produce joint lesions similar to clinical collagen diseases and the other pathological changes noted above, Selye now proposes that a factor "X" secreted by the pituitary selectively stimulates the secretion of mineralo-corticoids by the

adrenal cortex, while all the purified preparations of ACTH so far obtained are "gluco-corticotrophic". The possibility has been considered that this factor "X", may be similar to anterior pituitary growth hormone (77). Essentially, however, the concept of the diseases of adaptation still stands or falls on the assumption that stress eventually leads to an overproduction of mineralocorticoids whatever the mechanism.

It is obvious that if the pituitary-adrenal relationship is regulated solely by the mechanism proposed by Sayers, the overproduction of cortical hormone is precluded. It follows as a corollary that in the presence of excessive amounts of circulating cortical hormone stress should not be able to stimulate the anteriohypophysis to secrete ACTH.

These opposed interpretations of the existing experimental data prompted the re-examination of the possibility of inducing a hypophyseo-adrenal response to stress in the presence of a high corticoid concentration.

2. Experimental Work

The experiments were designed to establish if, in the presence of very high concentrations of desoxy-corticosterone acetate in the body fluids, stress could induce a release of ACTH (as indicated by the adrenal ascorbic acid method of Sayers (42,78,79).

The anesthetic effect of DCA (80,81,82), which is only obtained with excessive doses, served as an independent criterion to establish

that the amount of hormone administered was greatly in excess of the animals' requirements even under stress.

(a) Materials and Methods

(i) Ascorbic Acid Determination - Carruther's ascorbic acid method (83) is based on the rapid reduction of 2,6-dichlorophenolindophenol by ascorbic acid. The specificity of this reaction is increased by performing it at a pH between 2 and 3 and in the presence of mercury and cadmium salts, which inhibit the action of interfering substances. The dye was dissolved in a phosphate buffer solution (66 mM) which buffers it at pH 6.5 to 6.6,at which pH the dye is blue. To 5 ml. of this solution was added 1 ml. of a solution containing cadmium and mercuric chloride in a concentration of 0.001 M with respect to each metal ion. After the additon of 2 ml. of either tissue extract or standard ascorbic acid solution in 2% metaphosphoric acid, the pH drops to a value of 2.5 to 2.7 and the reduction takes place immediately. The unreduced dye has a light pink colour at this pH while the reduced leucobase imparts no colour to the solution. The addition of 6 ml. of phosphate buffer (pH 8) brings the pH of the mixture back to a value of 6.5 to 6.6 and the excess unreduced dye becomes blue. The optical density of this solution was determined with an Evelyn photoelectric colorimeter, setting the instrument with a blank solution prepared as above, except that pure 2% metaphosphoric acid replaced the tissue extract.

According to Carruthers, unknown amounts of ascorbic acid can be analyzed by determining the optical density of the blue dye before the addition of the metaphosphoric acid and again after reduction and

readjustment of the pH at a value of 6.5-6.7. The ascorbic acid equivalency of these readings is obtained from a percent transmittance-concentration curve, and taking into account the change in the volume of the solution the amount of ascorbic acid present can be calculated. The standard curves exhibited perfect linearity within the dilution range employed. Changes in the ascorbic acid equivalency of the dye due to age did not affect the linearity of the curve and recoveries were found to vary between 96 and 102 percent.

Since a large number of determinations were being done simultaneously it became of interest to determine if the presence of the heavy metal solution induced a change in the optical density of the dye upon long standing. Leaving this mixture at room temperature for as long as 5½ hours did not change the optical density of dye solutions of various concentrations; this allowed the preparation of the mixture well in advance of each experiment.

(ii) Experimental Plan - Thirty male albino rats weighing 80 to 100 gm. and previously fasted for 24 hours, were divided into three groups of 10 animals each. The first group received intraperitoneally 6 mg. of DCA dissolved in warm corn oil, and repeated doses of 2 mg. were similarly administered to each animal to maintain it in a state of anesthesia during the 6 to 7 hours that the experiment lasted. These rats were intermittently exposed to cold ($10 \pm 1^{\circ}C$) for one hour at a time, and between exposures they were allowed to recuperate for about half an hour. During the rest periods all animals recovering from the anesthetic effect received an additional dose of

2 mg. of DCA,and those animals that were profoundly narcotized were allowed longer rest periods. Most of the animals were exposed for a total of 4 hours and none for less than 3; the total dosage of DCA administered ranged from 12 to 14 mg. per animal. Group 2 received exactly the same cold treatment as Group 1 but no DCA was administered to it. Group 3 received no treatment and thus served as an absolute control. The results of this experiment are presented in Table 1 as Experiment 1. The same experimental plan was repeated using more acute conditions: the exposure to cold was lengthened to $4\frac{1}{2}$ hours and the total DCA dosage increased to 16 mg. per animal. Twenty rats were divided into two groups of 10 animals each: one group was exposed to low temperature and received DCA, the other group received no treatment. This is Experiment 2 in Table 1.

At the end of the experimental period the animals were sacrificed with chloroform, the adrenal glands rapidly removed and freed of the encapsulating fatty tissue by pressing the gland gently with the forefinger between two pieces of filter paper and popping the intact gland out. Each gland was transferred to a centrifuge tube containing a small amount of acid-washed sand,to facilitate maceration,and 5 mls. of 2% metaphosphoric acid,and was thoroughly ground with the aid of a mechanical stirrer. After centrifugation 2 ml. aliquots of the supernatant were taken for the determination of ascorbic acid.

(b) Results and Conclusions

In both Experiment 1 ($P < 0.05$) and Experiment 2 ($P < 0.02$) the difference in adrenal ascorbic acid between the DCA-treated cold-exposed group and the normal controls is significant.

TABLE I

Exp.	Group	No.of rats	Treatment	Adrenal Ascorbic Acid: mg/100 gm fresh tissue (mean ± standard error)	Adrenal Ascorbic Acid: % decrease from normal control
1	I	9	DCA + cold	320 ± 12.0	12
	II	10	Cold	320 ± 13.6	12
	III	10	Normal control	362 ± 12.3	—
2	I	10	DCA + cold	259 ± 15.0	18
	II	10	Normal control	317 ± 14.4	—

In Experiment 1, exposure to cold alone (Group II) brought about a decrease in adrenal ascorbic acid of the same order as the decrease found in animals which, simultaneously with the cold treatment, received DCA overdosage (Group I).

It should be mentioned that the cold-treatment did not significantly change the weight of the adrenals whether the animals received DCA or not, as shown in Table II.

TABLE II

Exp.	Group	No.of Rats	Treatment	Left Adrenal (mg)*	Probability
1	I	9	DCA + cold	5.2 ± 0.25	(I - III) $P > 0.4$
	II	10	Cold	5.4 ± 0.26	(II - III) $P > 0.3$
	III	10	Normal control	5.7 ± 0.16	
				Total Adrenal Tissue mg	
2	I	10	DCA + cold	11.3 ± 0.44	$P > 0.2$
	II	10	Normal control	12.2 ± 0.52	

* mean ± standard error

These results indicate that ACTH can be released from the pituitary of stressed animals even in the presence of a high DCA concentration in the body fluids.

3. Discussion

These findings are incompatible with the assumption that the discharge of ACTH by the pituitary is solely regulated by the cortical hormone concentration of the body fluids. On the other hand these experiments point to the possibility that in the presence of excessively high corticoid concentrations (as at times of very acute stress) the regulation of the discharge of corticotrophin may become relatively independent of circulating cortical hormone.

Sayers (23) has criticized these experiments, pointing out that the decrease in adrenal ascorbic acid which occurred in the untreated animals exposed to cold (Experiment 1, Group II) was surprisingly small. Dr. Sayers (43) reports a decrease of 50 per cent in adrenal ascorbic acid in rats exposed to a temperature of - 8 ± 1°C for one hour, however, in the same publication he also reports a decrease of only 14 per cent in rats exposed to a temperature of + 7 ± 1°C for one hour. This latter response is very similar to the values presented in Table I, where it is seen that exposure to a temperature of + 10 ± 1°C for 4 hours resulted in a decrease of 12 per cent in adrenal ascorbic acid. Furthermore, the low adrenal ascorbic acid response by the normal animals would indicate that the stress was not extremely acute. In spite of this, DCA overdosage did not prevent the release of ACTH by the anterior pituitary.

More recently, D'Angelo, Gordon and Charipper (85) have reported a similar inability to inhibit corticotrophic activity in stressed guinea pigs. These authors gave as much as 15 cc. of Upjohn's aqueous cortical extract (2.5 rat units per cc.) daily for 4 days to fasting guinea pigs, in an effort to inhibit the adrenal hypertrophy produced by inanition. Their results were negative either with this treatment or when 15 mg. DCA daily for 6 days was substituted for the extract. It must be pointed out that either of these treatments significantly depresses adrenal weight in the normal fed guinea pig. D'Angelo et al. concluded that the ineffectiveness of cortical hormones in preventing adrenal hypertrophy suggested that the catabolic reactions in starvation are such that ACTH secretion either cannot be appreciably inhibited, or that much greater amounts of cortical hormone than those effective in the fed animal are necessary.

A further objection raised by Dr. Sayers in the aforementioned review (23) is of a more serious nature: "The physiological significance of such an experiment remains in doubt. It would not be at all surprising to find that physiological levels of the cortical steroids have an inhibitory influence upon the pituitary, whereas toxicological doses, which produce general collapse of the organism, exert a toxic action on the adenohypophysis with release of stored ACTH".

The criticism that the experiments reported above are more pharmacological than physiological must be accepted. In this connection it is noteworthy that whereas the dosage of cortisone effective in the clinical treatment of the collagen diseases is of the order

of 100 to 200 mg. a day (65), Bergenstal and Huggins have demonstrated that adequate replacement therapy in the bilaterally adrenalectomized human can be obtained with as little as 25 mg. of cortisone per day (84). The actual physiological significance will remain in doubt until information is available concerning the titre of cortical hormone, in the body fluids of animals undergoing the various stages of the alarm reaction.

However, to this writer's knowledge, there is no experimental evidence to support the statement that steroid anethesia is the result of a "general collapse of the organism" or that overdosage with adrenocortical substances exerts a toxic action on the anterior pituitary leading to the release of stored ACTH.

C. Dietary Studies

1. Review of the literature

One of the earliest observations on the effect of dietary protein on the adrenal cortex was made by Fahr in 1912 (86,87). This investigator noted that the adrenals of rabbits fed a diet of milk and eggs doubled in size. The experiments of Orten (88) are cited by Tepperman, Engel and Long (87) as evidence that a high protein intake induces adrenal hypertrophy. In these experiments, Orten found that the adrenals of female rats receiving an 18 per cent protein diet underwent marked hypertrophy when compared to similar animals receiving 3.5 per cent dietary protein; although both groups of rats were on the same caloric intake. Increasing the caloric intake while keeping the protein content at 3.5 per cent did not result in adrenal hypertrophy. These observations indicate that the protein content of the diet contributes in determining the size of the adrenals.

In a review of the agents that cause adrenal hypertrophy, Tepperman, Engel and Long (24) concluded that any condition or agent that results in an increase of the products of protein catabolism induces an enlargement of the adrenals. They point out that many apparently unrelated conditions which stimulate adrenal hypertrophy, such as secondary hypermetabolism due to thyroxine administration, cold exposure, exercise, lactation, etc., all had in common a rise in the products of endogenous catabolism. Relative carbohydrate deprivation following starvation, phlorizin treatment or the administration of insulin also increases the breakdown of endogenous protein and results

in adrenal enlargement. Conditions which secondarily induce tissue breakdown (burns, anoxia, hemorrhagic or traumatic shock, infections, toxins, etc.) also result in adrenal hypertrophy. The origin of the protein catabolic products can be endogenous or exogenous, since a high protein diet also induces adrenal enlargement.

These authors put forth the explanation that excessive amounts of a substrate (S), derived either from the animal's own tissues or from dietary protein, requires the presence of cortical hormone for its catabolism and thus serves as a stimulus for adreno-cortical secretion. They further propose that this substance might act by, "mobilizing cortical hormone either by way of the end organ in which it is catabolized, or by acting directly on the adrenal cortex".

To elucidate this problem Tepperman, Engel and Long (87) studied the effect of high protein diets not only on the size of the adrenals but also on the gland's morphology, in an effort to find whether or not this dietary regime resulted in adrenal hyperactivity. Further, they compared the metabolic behaviour of animals on a high protein intake with that of animals receiving physiologically active cortical substances. The authors used three high protein diets: one containing ground beef (55% protein), and two made up of 75 and 60% casein. All three diets produced adrenal enlargement when compared to animals receiving laboratory chow (24.4% protein). The final body weights of these animals were not essentially different from the control groups, indicating that the various diets were capable of supporting adequate growth. Histologically the enlarged glands were characterized by

widening and lipoid depletion of the cortices. These changes conform to the descriptions given of actively functioning glands (89,90). These authors also report experiments in which they compared the fasting carbohydrate levels of meat-fed and chow-fed rats, together with normally-fed animals receiving cortical extract or Compound E. They state that "the changes in the meat-fed animals are in the same direction as those produced by the injection of cortical extract and Compound E". Both the fasting blood sugar and liver glycogen concentrations were higher in the meat-fed rats than in those receiving chow.

These metabolic observations and the histological studies suggest that concomitant with the enlargement of the adrenals there is an increase in hormonal secretion, which these authors interpret as being required for the metabolic disposal of substrate (S), a catabolic product derived from protein.

This interpretation has not gone unchallenged, and two types of objections have been raised. Firstly, some authors have reported that high protein diets do not induce adrenal enlargement (91,92,93,94). Secondly, adrenalectomized rats have been shown to survive and grow on a high protein diet (93), a fact that appears to be incompatible with the interpretation that the adrenal cortex is essential for protein metabolism.

It is not readily apparent why contradictory results have been found by different authors in a relatively simple experimental procedure. Benua and Howard (91) studied the effect of high protein diets on the size and histological appearance of the adrenal of the

mouse,and arrived at the conclusion that the protein content of the diet had no effect on the glands. One of the diets used by these authors was identical to Tepperman's Casein A diet (78% protein); however, no adrenal enlargement was observed, and body growth was normal. Another diet tested, containing 67% protein, behaved similarly, but a third dietary regime with 89% protein did not support body growth adequately in the unilaterally adrenalectomized mouse, although the adrenal weight per body weight ratio remained normal. The authors propose that since growth retardation was observed in the unilaterally adrenalectomized mouse but not in the intact animal, the operated animals were undergoing relative adrenal insufficiency. A second explanation put forth to explain this impaired growth was the possible toxicity of the diet. It is difficult to visualize either one of these situations not leading to compensatory adrenal hypertrophy, and therefore these hypotheses are not altogether satisfactory.

What this study did show clearly, was that the mouse did not undergo adrenal hypertrophy when fed Tepperman's high casein diet.

Leatham also failed to observe adrenal hypertrophy after feeding Tepperman's Casein A diet to rats of the Long-Evans strain (94). The control animals received a meat-supplemented diet with a total protein content of 32-33% which is about 10% higher than the control diet of chow used by Tepperman et al. In all but one of the experiments reported by these workers the animals studied were considerably larger (225-300 gm.) than the animals observed by Tepperman et al. It is interesting to note that,although the high protein diet did not induce

adrenal enlargement in the younger rats (90 gm. body weight), it caused greater renal hypertrophy than in the older animals, and also a significant rise of the blood non protein nitrogen not observed in any of the other experiments.

Such differences in experimental procedure as race, strain, and age of the animals, and differences in the control diets can hardly be called upon to explain these contradictory results in the light of Ingle's experiments (92,93).

This investigator studied the effect of various diets on adrenal size in the rat and on the capacity for survival of the adrenalectomized animal (93). He notes that the high protein diet (65% protein) resulted in slightly greater adrenal weights when compared to the action of a high carbohydrate regime. On close inspection of his results, however, it appears that there is no difference in adrenal weight between the animals fed the high protein diet and those fed a mixed diet (16% protein).

This author studied the effect of different test diets on survival following adrenalectomy. Normal rats were kept on four different diets (high protein, high carbohydrate, high fat and mixed) for a period of one week, then operated, and the same feeding regimes maintained until the time of death. The time of survival was the same in all four cases, which may be interpreted to mean that:

(1) The high protein diet did not require a higher level of cortical secretion than the other diets;

(2) The high protein diet was not particularly toxic to the animals. Adrenalectomized rats receiving the high protein diet and 1% sodium

chloride instead of drinking water grew as well as similarly treated animals receiving mixed or high carbohydrate diets. This is indirect evidence that the extra dietary protein does not require more cortical secretion to be adequately metabolized.

In a later publication (92) Ingle presents results of a comparative study done on Tepperman's Casein A diet (78% protein) and another high protein diet (57% casein). Unfortunately this latter diet is different from the one used in his previous experiments, and as a result it is not possible to establish too close a comparison between his reports.

He confirmed the results of Tepperman et al. by inducing adrenal hypertrophy with the Casein A diet and also with another diet having the same protein content, most of which was made up by lactalbumin. His own Casein C diet (57% casein) was not effective in inducing adrenal hypertrophy. In these experiments Ingle used rats of the same strain, sex and body weight as Tepperman's. The author is unable to give an explanation for these discrepancies but he points out some flaws in the interpretation given by Tepperman, Engel and Long (24), purporting to explain adrenal hypertrophy following various forms of stress by the common pathway of a substrate of protein catabolism.

Ingle's arguments against this hypothesis may be summarized as follows:

(1) The hypothesis does not distinguish between the action of the glucocorticoids and the cause of adrenal enlargement.

(2) All high protein diets do not result in adrenal hypertrophy.

(3) Adrenalectomized rats receiving sodium chloride survive and grow normally on a high protein diet.

Yet, the fact remains, that under certain not too well defined conditions, some protein diets elicit adrenal hypertrophy and that the numerous stressing agents mentioned above are characterized by increased protein catabolism. In his concluding remarks Ingle emphasizes that "none of these arguments against the hypothesis of Tepperman, Engel and Long are inconvertible.....(it) deserves further testing".

Other evidence of the action of dietary protein on the activity of the adrenal cortex has been presented by Selye and his group. These authors studied the effect of the composition of the diet on the pathological lesions elicited by the administration of crude anterior pituitary preparations, desoxycorticosterone acetate or the exposure to damaging stimuli.

It is questionable to what extent the experiments of Selye's group are comparable to those reported above. What the Montreal investigators refer to as a "high protein diet" contains 30% protein, which is only a slightly higher percentage than ordinary laboratory chow; furthermore it is between 1/2 to 1/3 of the protein concentration found in the "high protein diets" discussed above. Another point of dissimilarity lies in the sensitization to LAP that Selye's animals were subjected to. In some experiments on adult, male rats this was achieved by castration, unilateral nephrectomy and the replacement of drinking water by 1% Saline. Just how "physiological"

such experiments are, is a question of controversy.

It will be recalled that when sensitized rats are overdosed with DCA there results cardio-vascular lesions (48,49,50,51), hypertension (49,50), arthritic joint lesions (49,52) and nephrosclerosis (48,50,53,54). These pathological changes can also be produced by chronic treatment with a crude anterior pituitary preparation (LAP) (55,56) which also elicits hypertension (57). Further, damaging agents such as exposure to cold, formaldehyde injections, and exercise elicit hypertension and nephrosclerosis in sensitized animals (59). Finally, Selye contends that corresponding lesions found in clinical pathology are the result of overadaptation by a stressed individual.

Berman, Hay and Selye found that the nephrosclerosis and periarteritis nodosa,normally elicited within 40 days after the implantation of three 50 mg. pellets of DCA in female albino rats, were almost completely inhibited by the daily administration of 2-3 ml. of 25% dextrose solution by stomach tube (95). The fact that the animals were specifically sensitized to the damaging action of DCA by unilateral nephrectomy and by the daily administration of 2-3 ml. of 6% NaCl solution per os, adds weight to this observation. Throughout the experiment the animals received a prepared laboratory chow which according to the manufacturer contains approximately 25% protein. The glucose treatment also reduced the increase in cardiac weight but had no action on body growth.

In another experiment the authors sensitized rats to the damaging action of LAP by unilateral nephrectomy and the administration of 1% NaCl solution to drink instead of tap water. The animals received

30 mg. LAP subcutaneously per day and either one of two diets; one consisting of ordinary laboratory chow and the other a high-carbohydrate (70%) low-protein (15%) diet. This latter diet protected the rats from the fatal nephrosclerosis induced by the treatment in the animals receiving the normal, mixed diet.

The conclusion was drawn that if "the carbohydrate content of the diet is increased at the expense of the protein content, the resistance of the rat against nephrosclerosis and allied vascular phenomena is greatly augmented".

These findings were confirmed in experiments in which rats sensitized to the nephrosclerotic action of LAP were fed exclusively on the following diets: skeletal muscle, cardiac muscle, "Purina" fox chow, peas, lentils, corn, lima beans, or rice (96). The animals received daily subcutaneous injections of 20 mg. of LAP during 17 days. The degree of nephrosclerosis and the final weights of the kidney, heart and adrenal were roughly proportional to the protein content of the diets; and inversely proportional to the carbohydrate content. The magnitude of these pathological changes decreased in the order in which the diets are listed above.

Hay, Prado and Selye made a systematic study of these dietary effects in an effort to determine whether the lesions observed following LAP treatment were due to the high protein concentration or to the concomitant reduction in carbohydrate (97). They also introduced synthetic diets of well-controlled composition which were more closely comparable than those previously studied in their laboratory. When these diets, differing only in their relative

carbohydrate and protein content were fed, only those LAP-treated rats that consumed a regime containing 30% casein and 54% cornstarch developed nephrosclerosis. Rats similarly treated and kept on a 15% casein and 69% cornstarch diet did not develop these lesions. Evidence that the action of the diet is due to the protein concentration was derived from experiments in which the cornstarch was substituted by wheat starch, or 15% of it was replaced by an equicaloric amount of fat, without influencing the development of renal lesions.

It should be pointed out that in these and subsequent experiments from this group of workers, the male rats used were sensitized to the LAP treatment by castration, unilateral nephrectomy and the administration of 1% saline instead of drinking water.

The blood pressure of rats following LAP treatment was found to increase only when the animals were fed the 30% protein diet, while those on the 15% protein diet remained normotensive (57).

The high protein diet (30%) per se did not result in either nephrosclerosis, hypertension or marked adrenal enlargement; although it did have a renotrophic action. The administration of crude anterior pituitary preparation resulted in significant adrenal hypertrophy, irrespective of the protein intake, but the high protein diet induced a significantly greater increase in adrenal weight.

A similar study conducted to elucidate the rôle played by the diet on the pathological lesions following DCA overdosage, revealed that neither the hypertension nor the nephrosclerosis produced by this means were alleviated by a low protein diet (98).

The working hypothesis guiding these studies of Selye and his collaborators was that non-specific stress acting on the anterior lobe of the hypophysis through an unknown mechanism may be one of the stimuli which ultimately lead to the diseases of adaptation. Since the adrenals are essential in the pathogenesis of LAP-nephrosclerosis (58) and since the high protein diet accentuated adrenal hypertrophy due to LAP (57) it was tentatively concluded that the dietary protein acted in one of the following manners:

(a) inducing increased ACTH secretion;

(b) increasing the responsiveness of the adrenal cortex to the same dose of LAP; or

(c) potentiating the peripheral damaging action of the DCA-like steroids produced by the adrenal cortex.

This last possibility was ruled out by the experiments described above (98) in which it was shown that the protein content of the diet did not affect the nephrosclerosis or the hypertension produced by DCA treatment.

It was thought of interest, therefore, to attempt to elucidate the mechanism of action of the dietary protein as a regulator of adreno-cortical activity. This approach could throw light not only on the pathogenesis of the diseases of adaptation, but also on the relation-ship between the hypophyseo-adrenal system and stressful agents.

2. Experimental Work

The experiments reported below were directed at the following question: does the high protein diet render the adrenal cortex more responsive to pituitary stimulation or does it induce increased corticotrophic activity?

It was decided to investigate first whether the protein content of the diet had any effect on the adrenals of animals with endogenous corticotrophic hyperactivity. This was done by determining the rate of regeneration of the remaining adrenal after unilateral adrenalectomy in animals receiving high or low protein diets but not otherwise sensitized.

The action of the diet on the response of the hypophyseo-adrenal system to stress was investigated. The adrenal ascorbic acid depletion after exposure to cold was studied in rats following a preparatory feeding period on either one of the two experimental diets.

Finally, the responsiveness of the adrenal cortex to a fixed dose of exogenous ACTH was determined by the ascorbic acid method in hypophysectomized animals pretreated with either of the two diets.

(a) Materials and Methods

(i) Ascorbic acid determination:

The determinations of adrenal ascorbic acid were carried out as previously described under "Methods" in Section B.

(ii) Diets

The composition of the diets is given in Table 3. The mineral mixture is that of Steinbock and Nelson (99). Approximately 10 mg. alpha-tocopherol was given once a week to each rat by introducing it with a dropper into the animal's mouth. The bulk was contributed by "Cellu-flour"; the fat by "Crisco", a commercial hydrogenated vegetable fat. In preparing the diets, the amounts of starch and casein weighed were greater than the theoretical amounts

required; this was done to compensate for the moisture content of these constituents which was found to be approximately 5%.

TABLE 3

COMPOSITION OF TWO BASIC SYNTHETIC DIETS

(parts per cent)

Diets		I	II
Casein		15	30
Corn Starch		78	63
Fat		1	1
Cod liver oil		1	1
Bulk		1	1
Mineral mixture		4	4
Supplements:	Thiamine chloride	0.8 mg./100 gm. diet	
	Riboflavin	0.8 mg./100 gm. diet	
	Pyridoxine	0.8 mg./100 gm. diet	
	Ca pantothenate	4.0 mg./100 gm. diet	
	Nicotinic acid	1.0 mg./100 gm. diet	
	Choline chloride	100.0 mg./100 gm. diet	
	α-Tocopherol acetate	10.0 mg./rat/weekly	

The diets were prepared fresh every third day, made into biscuits and stored in the cold. The animals were allowed to eat the biscuits ad libitum.

(iii) Preparation of ACTH

ACTH was prepared from lyophilized cattle pituitary by the method of Fishman (100). This method was originally applied to hog pituitary and its application to bovine glands was not expected to give comparable yields, for this latter material has been reported poorer in ACTH (101).

Fishman's method uses crude prolactin as starting material [as prepared by White, Bonsnes and Long (102)] and accordingly this was first prepared. Three 50 gm. batches of lyophilized cattle anterior pituitary yielded a total of 4.48 gm. of crude prolactin. 2.5 gm. of this material extracted according to Fishman's procedure, yielded 94 mg. purified ACTH. According to this author a similar quantity of crude prolactin from hog pituitaries would yield about 325 mg. of purified ACTH. The low yield obtained was expected for the reasons mentioned above.

This purified preparation was tested by the method of Sayers and found to be biologically active. The results of these tests are reported below under sub-section (b).

(iv) Hypophysectomy

The pituitary was removed by the standard laryngeal operation essentially as described by Ingle and Griffith (103).* At the time the animals were sacrificed they were examined for pituitary remnants, and rats in which the operation had been incomplete were discarded.

(v) Adrenalectomy

The gland was removed through a lateral incision under ether or Nembutal anesthesia. The adrenal was freed of its enveloping fat layer and capsule by gently pressing on a piece of filter paper.

(b) Experimental Plans, Results and Conclusion

(i) Tests on purified ACTH

Twenty-four hours after hypophysectomy the left adrenal was removed under Nembutal anesthesia and the gland extracted for ascorbic acid determination. Immediately after the operation 10 µg. of the

* Thanks are due to Dr. Georges Masson for performing these operations.

ACTH preparation, dissolved in 0.5 ml. water (pH adjusted to 7) was administered into the jugular vein. Exactly one hour later the remaining adrenal was similarly removed under ether anesthesia and treated as above. The results are presented in Table 4.

TABLE 4

Test on Biological Activity of Purified ACTH

	Adrenal Ascorbic Acid mg/100 gm gland		
Rat	left gland	right gland	difference
1	533	456	-77
2	590	312	-278
3	584	495	-89
4	565	472	-93
5	470	420	-50
6	392	328	-64
7	502	375	-127
8	398	315	-83
9	472	456	-16
10	367	385	+18
11	408	416	+ 8
12	425	419	- 6

Of the twelve animals tested, 8 gave a marked response, while the remaining four were essentially unresponsive. The reason for this discrepancy is not known but it may be significant to note that animals 10-12 were tested with the same solution on the same day. Nevertheless it was concluded that the preparation was suitable for the experiments to follow.

(ii) Action of Dietary Protein on Adrenal Compensatory Hypertrophy

Forty male albino rats (80-100 gm.) were divided into four groups: the first two groups received diet I (15% casein), and the last two, diet II (30% casein). After a preparatory period of one

week, the left adrenal was removed from all animals of groups 2 and 4, while groups 1 and 3 were kept as controls. Five animals of each group were killed on the fifth day after adrenalectomy and the other animals on the tenth day. The remaining adrenals of the operated rats and the right adrenals of the control animals were fixed in "Suza" for 24 hours, dissected and weighed on an analytical balance. This experiment was repeated twice: the second time all the animals were sacrificed 10 days after unilateral adrenalectomy and in the third experiment, 15 days after the operation.

TABLE 5

EFFECT OF THE DIETARY PROTEIN CONCENTRATION ON THE DEGREE OF ADRENAL COMPENSATORY HYPERTROPHY

(Average and Standard Errors)

Treatment	Diet	No.of rats	Autopsy days after Adr-ect.	Final body weight gm.	Right adrenal weight mg.	Adrenal weight mg/100 gm. of body weight
Control	I	4	5 days	139 ± 3.8	10.0 ± 0.81	7.2
Adr-ect.*		5		128 ± 4.9	10.2 ± 0.39	7.9
Control	II	4		145 ± 5.8	10.9 ± 0.76	7.5
Adr-ect.		4		130 ± 6.6	11.6 ± 0.91	9.0
Control	I	11	10 days	155 ± 3.9	9.9 ± 0.36	6.4
Adr-ect.		11		154 ± 5.6	11.7 ± 0.63	7.2
Control	II	10		178 ± 4.1	9.8 ± 0.27	5.6
adr-ect.		13		173 ± 2.3	13.7 ± 0.50	8.1
Control	I	10	15 days	179 ± 3.9	9.7 ± 0.23	5.4
Adr-ect.		9		168 ± 4.4	10.6 ± 0.46	6.4
Control	II	8		187 ± 3.9	9.2 ± 0.32	4.9
Adr-ect.		9		170 ± 5.2	13.3 ± 0.45	7.8

* Adr-ect. = adrenalectomized.

The combined results of all three experiments are presented in Table 5. These findings indicate that the degree of adrenal compensatory hypertrophy was markedly affected by the composition of the diet. The proliferation of the remaining adrenal gland was more rapid in the animals receiving diet II (30% casein) than in those fed diet I (15% casein). This difference is significant ten or fifteen days following the operation, whether calculated from the absolute or from the relative weights of the glands (mg. adrenal tissue per 100 gm. body weight) as shown in Table 6.

TABLE 6

Significance of differences in average adrenal weight

5 days	10.2 ± 0.39) 11.6 ± 0.99)	p = 0.2
10 days	11.7 ± 0.63) 13.7 ± 0.50)	p = 0.02
15 days	10.6 ± 0.46) 13.3 ± 0.45)	p < 0.01

The regeneration curves shown in Fig. 1 are obtained by plotting the percentage increases in relative adrenal weights against the experimental periods following unilateral adrenalectomy. From this graph it can be seen that fifteen days after the operation the adrenals of the animals receiving the higher protein diet had regenerated almost 60%, while the glands of rats on the lower protein diet enlarged by less than 20%. It was not established whether the regeneration curve of adrenal tissue of the animals receiving the 15% casein diet would eventually reach the curve of the 30% protein group or whether it would permanently remain at a lower level.

INFLUENCE OF DIETARY PROTEIN CONCENTRATION
UPON THE REGENERATION-RATE OF ADRENAL TISSUE

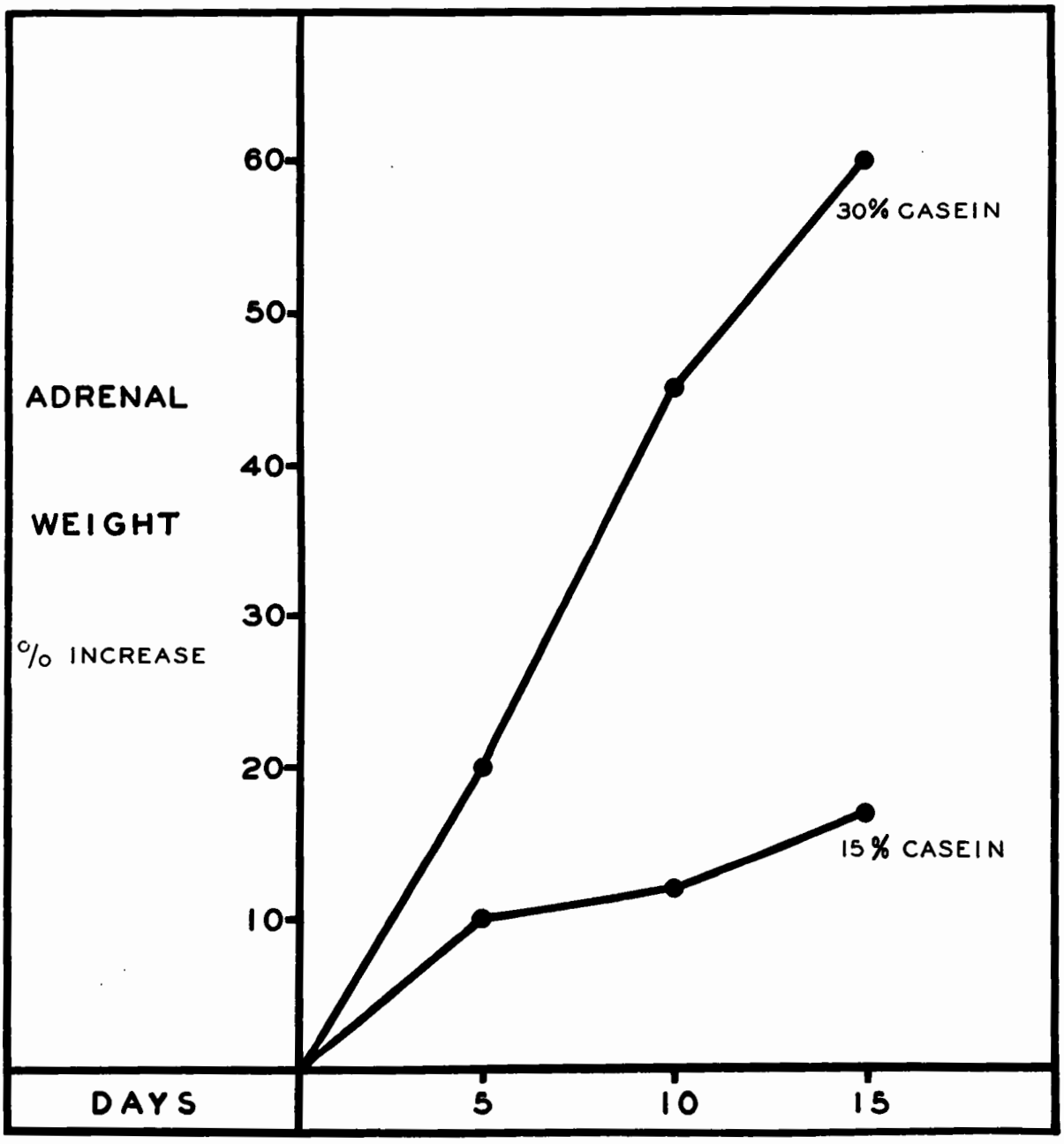

Figure 1

It is of interest that the final body weights of either control or operated animals on these two diets are practically the same, indicating that the animals on the low-protein diet were not deficient in nitrogen.

(iii) Influence of the Dietary Protein upon the Response of the Hypophyseo-Adrenal System to cold

Another group of experiments was designed to study the responsiveness to stress of the hypophyseo-adrenal system of rats receiving either high or low protein diet.

Four groups of male, albino rats (70-85 gm.) were fed the experimental diets during a period of 3 weeks. Groups 1 and 3 received the 15% casein diet, while groups 2 and 4 were fed the 30% casein ration. At the end of this time the animals were fasted for 24 hours and groups 1 and 2 were exposed to a temperature of $4 \pm 1^{\circ}C$ for one hour, while the remaining two groups were kept at room temperature as controls. All animals were then sacrificed with chloroform, and the ascorbic acid concentration of the adrenals was determined.

The same experiment was repeated subjecting the animals to a more intense stress ($0 \pm 1^{\circ}C$). These results appear in Table 7.

Another experiment was performed in order to ascertain the effect of the dietary protein concentration on the adrenal response to a given amount of exogenous corticotrophin. Two groups of adult, male, albino rats (70 to 85 gm.) were respectively fed diets I and II during a three-week preparatory period. The animals were hypophysectomized and 24 hours later, the right adrenal was removed under Nembutal anesthesia, and immediately after, 10 µg. ACTH was injected intra-

venously. One hour later the contralateral gland was also removed and the ascorbic acid concentration of each gland was separately determined. Care was taken to prepare the extract of each gland as it was extirpated. These results appear in Table 8.

TABLE 7

INFLUENCE OF DIET UPON THE RESPONSE OF THE HYPOPHYSEO-ADRENAL SYSTEM TO COLD

Group	Treatment	Diet	No. of rats	Adrenal ascorbic acid mg./100 gm. fresh tissue		Decrease in percentage
				Average ± S.E.*	Decrease from control level	
1	Cold (exposed to 4 ± 1°C. for 1 hr. before killing)	I	5	247 ± 5.2	32 ± 13.3	11.5
2		II	4	212 ± 15.0	73 ± 15.6	25.6
3	Unexposed controls	I	5	279 ± 12.2		
4		II	3	285 ± 29.2		
1	Cold (exposed to 0 ± 1°C. for 1 hr. before killing)	I	7	326 ± 5.5	48 ± 17.0	12.8
2		II	6	286 ± 8.7	148 ± 25.2	34.1
3	Unexposed controls	I	5	374 ± 16.0		
4		II	6	434 ± 23.8		

* S.E. = Standard Error.

Examination of Table 7 shows that exposure to cold caused a greater reduction of the adrenal ascorbic-acid concentration in animals receiving the high-protein diet than in rats fed the low-protein diet. If the decrease in adrenal ascorbic acid is expressed as the percentage of the control value, the difference between each

exposed group and its respective control is of the same order of magnitude in both experiments 1 and 2, in spite of the admittedly unexplained, low control values observed in the first experiment. It can also be seen that the dietary regimes per se did not affect the resting concentration of ascorbic acid in the adrenals.

These results suggest that the hypophyseo-adrenal system of animals receiving a high-protein diet gives a greater response to stress than that of animals fed a low-protein diet.

From these experiments it was not possible to establish whether dietary protein acted directly on the adrenal cortex or on one of the links between stress and this gland, such as the anterior pituitary or the hypothalamus. In order to study the effect, if any, of the dietary protein concentration on the responsiveness of the adrenal itself, the same amount of ACTH was administered to hypophysectomized rats receiving one or the other of the two experimental diets. The results of this experiment appear in Table 8.

TABLE 8

INFLUENCE OF THE DIET UPON THE RESPONSE OF THE ADRENAL TO EXOGENOUS ACTH

Treatment	Diet	No. of Rats	Adrenal ascorbic acid mg/100 gm. fresh tissue Average ± S.E.*		% Decrease after Injection
			Before ACTH (left adrenal)	After ACTH (right adrenal)	
Hypophysectomy; 10 µg.ACTH (after unilateral adrenalectomy & 1 hr.before killing)	I	6	450 ± 10.2	325 ± 18.8	30
	II	11	416 ± 18.8	320 ± 16.4	23

* S.E. = Standard Error

It can be seen that following the administration of 10 µg. ACTH to animals pretreated with diets I and II the decrease in adrenal ascorbic acid was not significantly different ($P = 0.1$). It appeared, therefore, that the dietary protein concentration did not modify the adrenal response to exogenous corticotrophin.

It is of interest to note that in this experiment, as in those described above, pretreatment of the animals with the experimental diets did not induce a change in the resting concentrations of adrenal ascorbic acid ($P > 0.1$). The total adrenal tissue was 11.6 mg. for the 15%-casein group and 12.0 mg. for the animals receiving the 30%-casein diet, and the difference was not significant ($P > 0.6$), indicating that the dietary regimes did not noticeably affect the non-stimulated gland. Finally, it should be mentioned that in three weeks, the animals on the low-protein diet grew from an initial average body weight of 73.7 to 167.9 gm., while the rats receiving the 30%-casein diet had an average initial body weight of 74.6 gm. and a final body weight of 176.3 gm. The difference in final body weights between the two groups was not significant ($P = 0.2$) which indicates that during the length of time that the experiment lasted, the low-protein diet did not result in impaired somatic growth.

To summarize the experiments described above, it can be concluded that the 30%-casein diet is more effective than the 15%-casein diet in stimulating some link in the chain of reactions leading from stress to cortical hyperactivity. This stimulation does not appear to consist in an increase in the responsiveness of the adrenal cortex itself. Furthermore cortical hypertrophy following unilateral adrenalec-

tomy is promoted by the higher protein diet, indicating again that the mechanism responsible for adrenal activity is stimulated.

III Discussion

The possibility that the decreased responsiveness of the hypophyseo-adrenal system of rats receiving the lower protein diet may be due to a state of malnutrition cannot be ignored. This possibility, however, seems unlikely as an explanation of the findings presented above, since during the relatively short feeding periods studied (a maximum of three weeks) the rats receiving the 15%-casein diet grew as well as those receiving twice that amount of protein. Furthermore, these concentrations of casein are not markedly different from the protein content of standard commercial diets for rats (approximately 25% protein) and therefore it may be advanced, that the animals were not subjected to an unphysiological nutritional regime.

The diets used by some earlier investigators are not comparable to diets I and II presented above, since in general "high protein" diets described in the literature have a protein content exceeding 50% and not infrequently levels as high as 80% have been studied (87, 91,92,93,94). For this reason it is felt that the present experiments throw no light on the action of "high-protein" diets on adrenal hypertrophy. On the other hand evidence is presented indicating that moderate alterations in the dietary protein content exert a marked influence on the normal animal's response to stress.

It is noteworthy that the animals studied in the experiments above were not sensitized to the action of stress or ACTH whether endogenous or exogenous. It will be remembered that studies on the

influence of the diet on the development of diseases of adaptation have generally been conducted under highly abnormal conditions; the animals being sensitized by castration, unilateral nephrectomy and high sodium chloride intake (57,95,96,97,104).

The data presented above can be analyzed in the light of Tepperman's theory of the adrenal enlargement due to high-protein diets (24). It can be argued that the 30%-protein diet requires a higher secretory level of cortical hormone for its metabolism and that the stimulus for this increased activity is provided by a product of protein catabolism. There is, however, evidence that this explanation does not conform to fact. Ingle (93) demonstrated that survival time after adrenalectomy was not affected by the protein content of the diet; an observation hardly compatible with Tepperman's theory.

It is, nevertheless, possible that a high concentration of circulating catabolic products derived from protein could serve as a stimulus to the hypophyseo-adrenal system. Tepperman, Engel and Long found that the administration of a casein hydrolysate to animals receiving a meat diet (55% protein) induced a hyperglycemia reaching 180 mg%, whereas the blood sugar of identically treated control rats did not change appreciably over a period of 4 hours after the parenteral administration of the hydrolysate (87). This effect could not be reproduced after adrenal demedullation and it was concluded that the action of the protein hydrolysate was mediated by the adrenal medulla as had previously been noted by Basiliou and Zell in 1931 (105).

More recently, other authors have put forth further evidence that some amino acids stimulate the adrenal. Todd and his coworkers

have shown that rats fed diets in which 10 to 15% of the carbohydrate is replaced by glycine, maintain higher liver glycogen levels during fasting than animals on a normal diet (106). This effect could not be elicited after adrenalectomy. The same authors have shown that following heavy doses of insulin the animals receiving glycine maintained higher muscle and liver glycogen stores, and underwent much less marked hypoglycemia (107). It was concluded that this effect could not be accounted for in terms of a direct conversion of glycine to glycogen. This interpretation is substantiated by experiments in which glycine labelled with C^{13} on the carboxyl group was fed to mice and the radioactivity of the expired CO_2 and liver glycogen determined. It was found that during the 16 hours following the administration of the amino acid, 50% of the C^{13} appeared as expired CO_2 and only a relatively small part of it was incorporated into liver glycogen. The conclusion was that glycine increases liver glycogen by promoting glyconeogenesis rather than by its direct conversion to carbohydrate (108).

A direct action on the adrenal medulla has been ascribed to glutamic acid by Weil-Malherbe (109), who observed the classical triad of adrenaline action: (1) fleeting rise in blood pressure

(2) fleeting rise in pulse rate

(3) rise in blood sugar

following the intravenous administration of a 25% neutral solution of glutamate to patients in hypoglycemic coma following insulin shock. Increase in the adrenergic activity of the blood was frequently observed after the administration of glutamic acid.

On the other hand there is ample evidence that epinephrine stimulates the hypophyseo-adrenal system. Vogt has shown that intravenous infusion of epinephrine induces immediate and long-lasting stimulation of the adrenal cortex (110). These effects were produced with dose levels which, she feels, occur under physiological conditions.

Many studies of the action of epinephrine on the pituitary-adrenocortical system have appeared in the literature since Thorn and his group showed that the number of circulating eosinophiles decreases after stress, and that the administration of small doses of epinephrine induced a similar decrease in eosinophile count, provided the pituitary and the adrenal cortex were intact (111). After adrenalectomy dogs and rats did not respond to epinephrine.

It may be tentatively put forth, that the effect of the high protein diet is mediated by the action of some constituent amino acid on the adrenal medulla, which in turn stimulates the pituitary-adrenal system via a hypothalamic humoral agent as suggested by Hume's experiments (112). If it is assumed that the blood amino-acid level contributes to the regulation of epinephrine secretion, then it must be concluded that the diets per se do not sufficiently disturb this level to induce increased activity of the hypophyseo-adrenal system, since no changes in adrenal size were detected in control animals not subjected to stress. When stressed, however, the animals receiving the high protein diet give a greater response. One possible explanation of this could be that stress further raises the blood amino-acid concentration to a threshold level that induces increased epinephrine

release. This critical concentration may be reached only when the pre-stimulation concentration of amino acid has been elevated by a high-protein diet. The evidence for this interpretation of the findings presented above is mostly indirect, but there is enough of it to suggest that protein catabolites may play a rôle in the regulation of the secretory activity of the adrenal cortex. The blood non-protein nitrogen of the rat has been found to reach a peak during the later part of the shock phase of the alarm reaction (113). Following X-ray shock (114), surgical stress (115) muscular exercise (116) and traumatic shock (117,118) the N.P.N. is raised. The blood amino-acid level has been found to increase after haemorrhage (119), protein starvation, administration of thyroxine and treatment with lipo-adrenal cortex extract (120). Conversely, after hypophysectomy or adrenalectomy the amino-acid level of the blood decreases (121).

Exposure to non-specific stressing agents (cold, muscular exercise, formaline, urethane) for short periods of time, elicited a greater response from animals receiving a high amino-acid diet than from rats fed a high carbohydrate regime (121). The high amino-acid diet resulted in greater adrenal enlargement and more extensive discharge of cortical lipids and lymphatic caryoclasia than the carbohydrate regime.

Attempts to identify the protein catabolite responsible for cortical stimulation have not been successful. The addition of commercial protein hydrolysates to 15%-casein diets to bring the nitrogen content to the level of the 30%-casein preparation, proved to be as effective as the high-protein diet in eliciting the nephro-

sclerotic and hypertensive actions of LAP on sensitized rats (122). These findings pointed to the amino acids of casein as the source of activity. However in LAP treated rats receiving a diet which contained 15% casein and one of the following amino acids in an amount corresponding to the nitrogen contained in a 30%-casein diet, nephrosclerosis did not ensue: glutamic acid, glycine, methionine, cystine, tryptophane, valine, histidine, lysine, leucine, phenylalanine, aspartic acid, isoleucine, norleucine and arginine (122).

In experiments in which all the dietary nitrogen was supplied in the form of pure crystalline amino acids, Henriques and her coworkers were able to elicit both nephrosclerosis and hypertension in sensitized rats receiving LAP (123). The nitrogen content of this diet corresponded to a 22%-casein diet and the control diet contained an enzymatic protein hydrolysate (Amigen) in a corresponding concentration. It is difficult to understand why this amino-acid diet should prove effective in eliciting the pathological changes induced by LAP, while a diet containing 15% amino acid plus 15% casein was not. A possible explanation for this discrepancy might be the manner in which these regimes were administered, since the synthetic diet containing only amino acids as a source of nitrogen was administered by stomach tube three times a day at regular 6-hour intervals while the other diet was fed ad libitum.

At any rate, the amino acids themselves appear to be responsible for the protein effect and other conditions that tend to increase protein catabolic products might be expected to result in increased cortical activity. Thus the effect of inanition in the guinea pig

has been found to result in adrenal hypertrophy, which is roughly proportional to the degree of body weight loss and duration of starvation (85). There are cytological manifestations of high secretory activity by the pituitary and in the absence of this gland cortical hypertrophy was absent. It is of great interest that the administration of high doses of cortical extracts which significantly depress the adrenal weight in the normally fed guinea pig did not prevent adrenal hypertrophy in starvation. The authors concluded that in starvation there is an increase in the secretion of ACTH by the basophils of the pituitary.

Conversely, subtotally nephrectomized rats fed an 8%-casein diet gave evidence of decreased ACTH secretion which presumably accounted for the normal blood pressure observed; a 30%-casein diet administered to similarly treated animals resulted in hypertension (124). Following the intraperitoneal administration of 0.2 mg. of epinephrine, the animals on the low protein diet showed a decrease in circulating eosinophiles that was only half the drop observed in the high-protein group; both groups responded equally well to ACTH. These experiments constitute further evidence that the dietary protein concentration has an effect on some part of the non-specific defense mechanism other than the adrenal cortex.

The experimental results presented above indicate that normal rats receiving a 30%-casein diet give a greater adreno-cortical response to stress than animals on a 15%-casein ration. This difference is not due to a change in responsiveness of the cortical tissue but probably the result of an increase in ACTH release. The

mechanism whereby the higher dietary protein concentration brings about this activation is not clear, and little more than speculation is possible until more is known about the physiological pathway leading from non-specific stress to hypersecretion of ACTH by the pituitary.

Part II

STUDIES ON THE GLYCOGENOLYTIC HYPERGLYCEMIC HORMONE OF THE PANCREAS

A. Introduction

There is a growing body of evidence suggesting that the so-called Hyperglycemic Glycogenolytic Factor (HGF) of the pancreas may be regarded as a hormone concerned with carbohydrate regulation. When the present study was begun, one of the obstacles to further research on the subject was the lack of a method to obtain HGF from the pancreas in reasonably good yields. A second point of interest concerning this factor was the possibility of establishing whether it played a rôle in the physiology of carbohydrate metabolism in man, or was merely a substance with interesting pharmacological properties.

Accordingly the present study is divided into two parts:

(a) attempts to extract HGF from a waste product of insulin preparation and -

(b) attempts to determine if this factor is present in the urine of normal individuals, with a view to the possibility that changes in the rate of excretion might throw some light on disturbances of carbohydrate metabolism.

It will therefore be convenient in reviewing the literature to deal first with reports concerned with hyperglycemic extracts derived from the pancreas, and follow this with a review of the work published on hyperglycemic preparations derived from urine.

B. Review of the Literature

The earliest report that a pancreatic extract produces hyperglycemia was published by Macleod (125) in 1922. The following year Gibbs, Root and Murlin (126,127) demonstrated the same effect using a differently prepared extract. In the light of present knowledge

it is questionable whether either of these preparations produced hyperglycemia by virtue of their HGF content. Macleod used the fasted rabbit as test object, and workers on the field are agreed that without adequate glycogen stores HGF does not elicit hyperglycemia. Murlin et al. obtained hyperglycemic preparations not only from the pancreas, but also from muscle and even from brewer's yeast, a fact which greatly detracts from the specificity of their pancreatic preparations.

Other investigators prepared hyperglycemic materials from the pancreas, but in general the extracts were so impure, or the test conditions such that their work is of questionable value.

By 1926, however, several authors agreed that commercial insulin preparations induced transitory hyperglycemia before lowering the blood sugar (128,129).

The first extensive experimental studies on this subject were those of Bürger and his associates. In 1928 this author observed that the administration to dogs of 0.4 units of insulin per kilogram of body weight consistently induced transitory, moderate hyperglycemia prior to lowering the blood sugar concentration. The highest blood sugar level was reached about 10 minutes after the injection and the hyperglycemic phase began 20 minutes after treatment (130,131). Both the time relationships and the moderate rise in blood sugar are known today to be characteristic of purified HGF. Further study revealed that amorphous commercial preparations of insulin invariably showed this hyperglycemic activity, but not some types of crystalline insulin (132).

This last observation, and the demonstration that insulin activity was destroyed by heat in an alkaline solution, while the hyperglycemic effect persisted after the alkali treatment (132), convinced Bürger that the two effects were due to two distinct substances.

Bürger observed that in the dog, the hypoglycemic effect of insulin could be lessened by removing from the circulation certain muscle masses (130). Since these experimental conditions did not affect the hyperglycemic phase, the liver was next investigated. In a series of cleverly designed experiments this author was able to show that the site of action of the hyperglycemic component of commercial insulin was the liver. Administration of crude insulin into the portal vein of dogs produced a more marked hyperglycemic response than when administered by other routes. Following the injection of insulin he observed a decrease of liver glycogen which was greater when the initial glycogen concentration was high (133). Neither adrenalectomy (134,135) nor denervation of the liver (136) interfered with this response, but an intact hepatic circulation was essential (136). Further evidence that the liver plays an essential rôle in this effect was afforded by the demonstration that patients suffering from cirrhosis of the liver showed a much lower hyperglycemic response than normal human subjects (137).

In later experiments Bürger found that depancreatized dogs did not respond to the administration of pancreatic hyperglycemic preparations, presumably because the liver glycogen is already depleted (138).

His various experimental studies led Bürger to conclude that the action of the hyperglycemic substance derived from insulin was due to its effect on liver glycogen, and that the blood sugar rise could be accounted for on the basis of glycogenolysis and release of glucose into the blood stream.

Some of the experiments reported by this investigator have been justifiably criticized by Stewart (139), who pointed out that the studies of liver glycogen changes in dogs were performed under such unphysiological conditions as deep narcosis and surgical trauma. An inconsistency on Bürger's part was the use of fasted rabbits as test objects after demonstrating the importance of high glycogen stores. Nevertheless the bulk of the German author's work is well planned and his results all point towards the same conclusion. It is regrettable that contemporary investigators in general have ignored his work, particularly since so many of the more recent studies have been but confirmations of Bürger's observations.

Although Bürger made the stimulating suggestion that the hyperglycemic effect was brought about by a true pancreatic hormone, possibly related to the etiology of diabetes mellitus (140), relatively little work was done in this field from 1935 to 1945.

Two important publications appeared in 1945 giving a new line of evidence for the existance of a pancreatic factor that opposes the action of insulin on the blood sugar concentration. R.-Candela demonstrated that the insulin requirement of the alloxan-diabetic dog is not affected by ligating the pancreatic duct but is markedly reduced if, following ligation, the remaining pancreatic tissue is

surgically removed (141,142). The animals were rendered diabetic by the administration of 70 mg. of alloxan per kilogram of body weight. When the diabetic state had become stabilized the blood sugar level was regulated with crystalline insulin administered three times a day before each meal. Several weeks later, when the diabetes was under control, the pancreatic ducts were ligated and sectioned. Sufficient time (6 to 8 weeks) was allowed for fibrotic degeneration of the pancreas before proceeding to remove this organ. The author reports that this operation was frequently very difficult because the pancreas, now a hard, fibrotic cord, tenaciously adhered to the duodenum. Ligating the pancreatic ducts had no effect on the insulin requirement but removal of the pancreatic remnant decreased the insulin requirement by 20 to 50%. This observation led R.-Candela to postulate the presence of an anti-insulin substance in the alpha cells of the islets of Langerhans. In a later publication this author repeated these experiments following the same plan. The dogs were kept on a diet of bread and raw pancreas, which allowed the animals to maintain their body weights reasonably well. The decrease in insulin requirement following pancreatectomy was again observed (142).

Simultaneously Thorogood and Zimmerman presented similarly conducted experiments in which they arrived at essentially the same conclusions (143). These authors showed that following pancreatectomy in the alloxan-diabetic dog the insulin requirement is decreased by 55 to 65%; glycosuria is decreased and ketonuria is very markedly raised. When the animals were not treated with insulin, survival after alloxan was much longer than following

pancreatectomy. Ligation of the pancreatic ducts did not affect the course of alloxan diabetes. Although the authors arrived at the conclusion that the pancreas secretes an anti-insulin substance, the evidence they present that the alpha cells are responsible for this function is not as direct as R.-Candela's, because technical difficulties prevented them from removing the pancreatic remnant after duct ligation.

These experiments have been criticized by Mirsky et al. (144), who point out that there are marked nutritional differences (and necessarily metabolic differences also) in the alloxan-diabetic dog before and after pancreatectomy. He found a marked decrease in glycosuria following the operation when the animals were kept on the same food intake and insulin therapy; however the body weight did not increase, as might be expected, during a post operative period of 40 days. This finding led Mirsky to analyze the carbohydrate and protein content in the faeces, and his results revealed that the carbohydrate excretion was not altered by the operation; the output of protein, however, was increased from 15.1% to 39.2% of the protein intake. This difference was statistically significant and the authors interpret it to mean that decreased glycosuria following pancreatectomy is the result of reduced absorption of carbohydrate precursors.

Furthermore, fasting induced a much greater rise in blood ketone bodies and in glycosuria after pancreatectomy and the blood glucose concentration was maintained at a higher level. The conclusion was drawn that pancreatectomy aggravates the severity of the diabetic

state of the alloxan-treated dog and that the decreased glycosuria found in the fed depancreatized animal receiving insulin is the result of decreased absorption of carbohydrate precursors, which masks the aggravation of the endogenous metabolic derangement. The ability of the fasting, alloxan-diabetic dog to form ketone bodies at a rate similar to that observed in the depancreatized animal was shown by administering phlorhizin to the alloxanized animal. This treatment was followed by a rise in urine sugar and in blood ketones quite similar in magnitude and rate to the changes observed in fasting depancreatized animals. The explanation is advanced that phlorhizin by depleting the liver glycogen stores, raises the production of ketone bodies. According to Mirsky and his collaborators the exacerbation of the diabetic state following pancreatectomy is probably due to the removal of functionally-active beta cells not previously destroyed by the alloxan treatment.

R.-Candela has answered this objection by re-stating his findings that in the alloxan-diabetic dog, ligation of the pancreatic ducts did not alter the insulin requirement, and he therefore concludes that "the pancreatic enzymes do not play a rôle in the reduced insulin-requirement of the alloxan-diabetic dog after pancreatectomy". The author proceeds to state his conclusion that the pancreas produces some factor which is responsible for the difference in insulin requirement (145).

Since 1947 interest in this problem has been renewed and several investigators have turned their attention to it. de Duve and Hers, restudying the action of insulin on glycogen stores, came to the conclusion that some commercial insulin preparations produced hyperglycemia

by inducing liver glycogenolysis (146,147). Since the hyperglycemic effect is a very transitory one, these authors found that it could best be elicited by continuous infusion. Their results agreed with those of Bridge who had used a similar experimental arrangement (148), and with those of Wichels and Lauber, who were able to produce prolonged hyperglycemia in rabbits by injecting repeated small doses of commercial insulin at short intervals (149,150). de Duve et al. pointed out that one brand of Danish commercial insulin ("Novo") did not show the initial hyperglycemic phase, and they therefore concluded that this preparation was free of the contaminant responsible for this effect.

These findings were confirmed shortly afterwards by Olsen and Klein (151), who in addition found that when such insulin preparations are given intramuscularly the hyperglycemic effect does not appear.

In 1948 Heard, Lozinski, Stewart and Stewart (152) reported that an extract derived from the first isoelectric precipitation of insulin [in the method of Romans, Scott and Fisher (153)] induced hyperglycemia and glycogenolysis in fed rats. These authors also present results of <u>in vitro</u> experiments showing that rat liver slices incubated in Ringer-phosphate medium release 20 to 30% more glucose into the medium in the presence of their extract. Since extracts of muscle or liver prepared by the same procedure failed to display these properties, the authors concluded that a specific pancreatic substance was responsible for their observations.

Simultaneously there appeared the first of a series of studies by Sutherland and his collaborators (154). These authors showed that purified amorphous insulin and some crystalline preparations contain

a factor which stimulates glycogenolysis in the isolated liver slice suspended in phosphate-buffered saline. As little as 6 µg. of a crystalline zinc-insulin preparation gave a marked increase in the glucose output of rabbit liver slices; however, "Novo" insulin, purified by the manufacturer, did not show this effect even in doses ten times higher.

The glycogenolytic factor, unlike insulin, was found to be resistant to alkali or cysteine treatment. It is non-dialyzable and precipitable by trichloroacetic acid in native insulin, but only partially precipitable after alkali treatment. The authors suggest that it may be a protein, since its action disappears following treatment with trypsin. Intact cellular structure appears to be essential for its activity since grinding or freezing and thawing the test liver tissue prevents the appearance of increased glycogenolysis.

In a later publication Sutherland and de Duve studied the distribution of the hyperglycemic factor in the pancreas of normal and alloxan-diabetic animals (155). They succeeded in extracting a hyperglycemic glycogenolytic substance from the pancreas of both normal and alloxan diabetic dogs. In the dog, the distribution of the active substance followed closely the distribution of the islet tissue, being highest in the so-called "tail" of the pancreas and lowest at the "head" of that organ.

The fetal calf pancreas contains relatively small amounts of digestive enzymes and histologically the ratio of islet tissue to acinar tissue is much higher than in the adult; the insulin content

is also higher in the fetal gland. As might be expected, the hyperglycemic activity of extracts of fetal calf pancreas proved to be considerably higher than that of similarly prepared extracts derived from adult tissue. Further evidence that the hyperglycemic activity is associated with the endocrine tissue was derived from the observation that in the sclerosed pancreas following duct ligation, the concentration of hyperglycemic material increased. It was observed that extracts derived from the pancreas of alloxan-diabetic rabbits contained normal amounts of the glycogenolytic factor, and produced prolonged hyperglycemia without subsequent hypoglycemia when administered to normal rabbits. All this evidence led the authors to suggest that probably the alpha cells were responsible for the elaboration of the hyperglycemic substance.

The glycogenolytic factor was also found in the upper three-fourths of the gastric mucosa of the dog, but not in the pyloric mucosa; small amounts were present in the duodenum and ileum. The factor could not be demonstrated in seventeen other tissues tested. In short the material seems to be present only in pancreatic islet tissue and in parts of the gastric mucosa. Certain argentophil cells are particularly numerous in the fundus mucosa of the dog, whereas the pyloric mucosa and the rest of the gastrointestinal tract is relatively free of them (156). Furthermore, it has been suggested that certain pancreatic cells that stain with silver are identical with the alpha cells (157,158). Sutherland and de Duve concluded that "the distribution of the glycogenolytic factor is apparently closely related to the distribution of these argentophil cells".

In these studies Sutherland et al. prepared their extracts following one of the procedures used to purify insulin (159) and the glycogenolytic activity was measured in vitro using rabbit liver slices. The assay was carried out by incubating slices from the livers of well fed rabbits in phosphate-buffered saline; the incubation was carried out in open tubes with air as the gas phase, shaking at 110 oscillations per minute in a water bath at 37°C. After 45 minutes the reaction was stopped by the addition of barium hydroxide followed by zinc sulphate and the filtrate analyzed for glucose. The authors claim that the response to different concentrations of the factor was found to be graded and reproducible, a fact which allowed the use of the test as a quantitative assay. The results were expressed as per cent of maximal stimulation after subtracting the glucose output of untreated slices. It is unfortunate, however, that the authors did not present replicate determinations, since liver slices are notoriously inconsistent in their metabolic responses.

Using a modification of this assay Audy and Kerly have found that the glycogenolytic activity of pancreatic extracts from rabbit, rat, cat, guinea pig and ferret are of similar potency (160). Further evidence was presented to bolster the suggestion that the islets secrete HGF, by demonstrating its presence in the endocrine tissue of the teleost Lophius piscatorius, a species in which this tissue occurs separate from the acinar tissue.

The methods of purification of HGF have, as a rule, followed the same pattern as those used to purify insulin. In fact, most

investigators have used insulin preparations at various stages of purification as starting material.

Sutherland, Cori, Haynes and Olsen (161) have purified the HGF from amorphous insulin and obtained fractions which were 10 times as active as the original material by the rabbit liver slice test. Alkali-inactivated insulin was fractionated with ethanol, and at a concentration of 83% alcohol a very active fraction separated after standing one day in the cold. Alcohol fractionation combined with isoelectric prcipitation (pH 6.4) gave a very active material which showed half maximal activity at a dose of 0.9 µg.; this represented a tenfold increase in potency over the starting material. Glycogenolytic fractions were prepared from the dog's stomach by subjecting it to acid-alcohol extraction followed by ammonium sulphate fractionation; however, these fractions were considerably less pure than the preparations referred to above.

Alkali-inactivated insulin was fractionated with trichloroacetic acid; a considerable part of the factor was precipitated by 1.5% trichloroacetic acid and the remainder when the acid concentration was raised to 4.5%. This was also true of gastric mucosa. The present writer independently made similar observations working on a different starting material and these findings were reported (162) at about the same time as those of Sutherland.

The "15% sodium chloride precipitate" of crude insulin in the procedure of Romans, Scott and Fisher (153) has been used as initial material in the purification of HGF (163). It was found that the following fractions showed hyperglycemic activity:

(a) 31% sodium chloride insoluble, non-dialysable

(b) 80% ethanol soluble fraction derived from (a)

(c) 80-90% ethanol insoluble fraction derived from (a)

(d) 2.5% trichloroacetic acid soluble derived from (c)

(e) 2.5% trichloroacetic acid insoluble derived from (c)

All the fractions except the trichloroacetic acid insoluble one exhibited insulin activity, a fact which suggests that this last procedure may be adequate for the separation of HGF from insulin.

Foà et al. have presented a procedure to extract HGF from pancreas, not based on any of the common methods used to extract and purify insulin (164). Lyophilized pancreas was treated with liquid ammonia to extract the active material, and the insulin in the extract was inactivated with cysteine. When dissolved in liquid ammonia a given amount of insulin can be inactivated by 1/40 of the cysteine required to destroy it when this hormone is dissolved in water. Foà and his collaborators feel that this milder form of extraction might rule out the possibility that HGF is a cleavage product of insulin. Similarly prepared extracts of liver or kidney possessed no hyperglycemic activity, but a preparation derived from stomach tissue was active. Besides the fact that HGF is extractable by liquid ammonia this publication adds little to our knowledge of this substance. The investigators state that this type of extraction was undertaken because previous attempts at purifying HGF made use of either the alkali or the high cysteine inactivation of insulin, and both these operations have undesirable features of their own: cysteine induces mild hyperglycemia, and they consider alkali treatment drastic.

From the communications reviewed above, it can be seen that modern investigators have conclusively shown that insulin-free pancreatic extracts stimulate liver glycogenolysis *in vivo* and *in vitro*, which results in increased glucose output by this tissue. The mechanism of this action has been elucidated by Sutherland et al. (154). These authors made the generally accepted assumption that the secretion of glucose by the liver involves the following reactions:

glycogen + PO_4 → glucose -1-PO_4 → glucose -6-PO_4 → glucose.

From this it followed that there were three possible sites of action for HGF (at the position of each arrow in the scheme above). *In vitro* studies using liver slices showed that the presence of phosphate in the medium was necessary to elicit the full glycogenolytic effect of HGF. The authors concluded that this observation "points to an action on the phosphorylase system". This conclusion seems unwarranted by these experiments, since stimulation of either of the two reactions following implies an increase in the conversion of glycogen to glucose -1- phosphate, which can only take place if sufficient inorganic phosphate is available to the tissue. However when glucose -1- phosphate was added to the slices, the tissue was found to be quite capable of dephosphorylating it in addition to the endogenous ester derived from glycogen. This suggests that phosphorylase, rather than phosphoglucomutase or phosphatase, is limiting the over-all rate of glucose formation from glycogen. Furthermore, HGF had no effect on the dephosphorylation of glucose -1- phosphate, which was shown to accumulate in

the tissue under the action of HGF (165). In the light of this additional evidence an increase in glucose output in slices incubated with HGF must, therefore, be due to a stimulation of the phosphorylase system.

In later experiments Sutherland showed that epinephrine also increases glycogenolysis of liver slices in vitro by stimulating this same enzyme system (166).

Further investigation of the phosphorylase reaction showed that the concentration of active enzyme is increased by HGF or epinephrine (166). This was demonstrated directly by measuring the phosphorylase activity of homogenates and extracts prepared from slices preincubated with or without these agents. When liver slices were preincubated as previously described for 20 to 30 minutes, the phosphorylase activity decreased considerably. This decrease was less pronounced when the slices were incubated in the presence of HGF or epinephrine. When either of these substances was added to preincubated liver slices, the phosphorylase activity was restored to the original level. Epinephrine was shown to increase the amount of active phosphorylase in muscle.

It might be inferred that HGF has no effect on muscle phosphorylase since the injection of purified HGF into the rabbit induces no change in the lactic acid concentration in the blood, while a marked hyperglycemia was observed. Vuylsteke et al. have reported a decrease in blood lactate following the administration of HGF, but they suggest that this effect may not be a specific response since it can be elicited by induced hyperglycemia (167).

The action of a pancreatic hyperglycemic preparation on muscle glycogen has been studied *in vitro* (168). Rat hemidiaphragms were incubated in the presence of insulin with and without the hyperglycemic material, according to the technique of Stadie et al.(169). The authors report that the hyperglycemic fraction decreased the glycogen deposition induced by insulin by as much as 75%. This publication deserves severe criticism on several accounts. First, no results obtained with untreated control tissue are presented for the purpose of comparison; second,the action of the hyperglycemic material *per se* on muscle glycogen was not investigated,and as a result the reader is left wondering if the preparation may not inhibit insulin by exerting some chemical action that inactivates this latter hormone. Little detail is given regarding the procedure followed in the preparation of the extract or its chemical properties, and therefore it is not possible to compare this material with preparations described by other investigators.

The concentration of ketone bodies in the blood stream has been found to be influenced by the pancreatic hyperglycemic substance. Thorogood and Zimmerman (143) showed that following pancreatectomy in the alloxan-diabetic dog, ketonuria is markedly raised, an observation that might be interpreted to mean that the decrease in insulin requirement and ketone body formation are interrelated. The administration of HGF has been shown to cause an increase in the blood concentration of ketone bodies in fasted pancreatectomized dogs (171). The rise in ketone concentration paralleled the changes in blood sugar level, although the hyperglycemic phase was not very

marked. The authors interpreted this parallelism as resulting from the depletion of hepatic glycogen stores, but admit that the rapidity with which hyperketonemia occurs is surprising. It is extremely difficult to reconcile these experiments with the observation that in the absence of the pancreas there is increased formation of these metabolites. As previously mentioned Mirsky et al. (144) observed a sharp rise in blood ketone levels when depancreatized dogs were fasted, while alloxan-diabetic animals deprived of food registered only a moderate increase in blood ketones. It is Mirsky's opinion that residual beta-cell tissue surviving the alloxan treatment is responsible for this difference.

The situation is further confused by Foà's experiments demonstrating not only the hyperketonemic effect of purified HGF, but also the presence of a hypoketonemic substance in crude extracts of pancreas with hyperglycemic activity (172). It is probably too early to draw any valid conclusions on this intricate phase of the metabolic behaviour of HGF.

Apart from the metabolic properties described above, HGF has been studied from the point of view of its relation to growth hormone. Bornstein et al. (173) reported that portal blood from normal cats rendered diabetic by the administration of growth hormone, produces a hyperglycemic response in alloxan-diabetic hypophysectomized-adrenalectomized rats, (ADHA rats). Similarly, portal blood from ADHA rats treated with growth hormone was found to induce hyperglycemia in recipient ADHA rats. The authors tentatively conclude that under the stimulus of growth hormone the pancreas releases HGF,

since the direct administration of growth hormone to recipient ADHA animals did not elicit hyperglycemia. The physiological significance of these experiments cannot be fully assessed; the ADHA rat is a sensitive test object, but a very unphysiological one. It should be recalled that in metahypophyseal diabetic dogs, the diabetes is associated with destruction of the beta cells of the pancreatic islets, while other parts of the pancreas may be left largely untouched (174). At present, therefore, it does not appear likely that the diabetogenic effect of growth hormone could be explained solely through its action as a stimulator of HGF secretion.

Another study attempting to establish a relationship between growth hormone and HGF, deals with the observation that HGF increases the width of the epiphyseal cartilage of the hypophysectomized rat, when assayed by the tibia test used for the bioassay of pituitary growth hormone (175). The activity of HGF preparations by this assay was found to parallel their glycogenolytic potency <u>in vitro</u>. The author reported three experiments,in two of which increasing the dose of HGF did not result in a greater response, a behaviour which is different from that of growth hormone. It is admitted that other hormones (thyroxin, prolactin and testosterone) also share this property of inducing a limited widening of the epiphyseal cartilage under the conditions of the test. From this information it appears that the effect may well be a non-specific one. In a third experiment in which higher doses were used, the response increased with the dose; however, the animals tested were too few for statistical analysis. Furthermore, the dose levels tested in this last experiment were very toxic (mortality rate 20-60%), a fact which greatly detracts from the

physiological significance these experiments might have. Therefore, on the basis of the evidence available at present, the theory that the effects of growth hormone are mediated by HGF released from the pancreas, must be regarded as unproven.

The evidence presented above shows that it is possible to separate a hyperglycemic glycogenolytic substance from pancreatic tissue. The experiments of R.-Candela (141,142) and Thorogood and Zimmerman (143) suggest that such a factor may be present in functionally active islet tissue in situ. But, rigorously considered, these investigations do not establish HGF as a physiological entity with bearing on the animal's metabolism. Such evidence did not appear until 1949 when Foà et al. presented what is undoubtedly the most important claim of HGF to the family of hormones (176). By cross-circulation experiments in dogs, these authors showed that the blood sugar of a dog receiving pancreatic blood from a normal donor injected with glucose, decreases sharply, reaching a minimum level in 30 to 60 minutes and returning toward the normal concentration about 2 hours after the anastomosis is disconnected. This observation is consistent with the hypothesis that a rise in blood sugar stimulates the secretion of insulin and by so doing regulates itself through a negative feed-back mechanism. When in the same experimental arrangement the normal donor was replaced by an alloxan-diabetic animal, the blood sugar of the recipient dog rose significantly, reaching a maximum value in 30 minutes. If in either experiment the recipient dog received blood from the mesenteric vein of the donor, instead of pancreatic blood, the effects described did not appear. These

authors concluded that the hyperglycemia elicited in the recipient dog by pancreatic blood from an alloxan-diabetic donor, indicated the presence of a hyperglycemic agent secreted by the alloxan-resistant pancreatic tissue.

Further studies using the same experimental arrangement showed that when the donor animal received insulin, and the recipient dog received pancreatic blood, the latter's blood sugar rose, while the donor's glucose level fell. When the donor received purified HGF, its blood sugar concentration increased and the recipient's decreased (177).

When the cross-circulation was established between the donor's mesenteric and the recipient's femoral veins, the administration of insulin to the donor resulted in hypoglycemia in both animals.

These experiments support the theory that, in vivo, a decrease in the blood sugar level of the donor stimulates the secretion of HGF by its own pancreas; the HGF poured into the pancreatic vein induced hyperglycemia in the recipient dog. Conversely when the donor received HGF, the resulting hyperglycemia stimulated its pancreas to secrete insulin, which in turn produced hypoglycemia in the recipient animal.

Foà and his collaborators conclude that HGF is a true pancreatic hormone and that, like insulin, its secretion is regulated by the concentration of glucose in the blood stream.

The question naturally arises: what is the clinico-pathological significance of the hyperglycemic hormone of the pancreas? It is generally accepted that the juvenile or labile diabetic represents

a distinct clinical entity from the old, obese diabetic patient. Lozinski (178) has suggested that the etiology of the pathological manifestations in the first type might be absolute deficiency of insulin; whereas, the second group might well be suffering from hypersecretion of HGF.

This hypothesis is strengthened by the findings of Bornstein and Lawrence (179) who determined the insulin content of the blood of patients from both these types of diabetics, using a very sensitive insulin test (180). Blood samples were taken from patients 2 hours after the oral administration of 50 gm. of glucose and injected into alloxan-diabetic-hypophysectomized-adrenalectomized rats. In five young patients with the clinical features of labile diabetes (hyperglycemic, glycosuric, emanciated, ketotic, insulin-dependent) the assay showed no available insulin in the plasma. In 5 middle-aged, obese women whose hyperglycemia and glycosuria (no ketosis) could be controlled by an adequate diet without insulin therapy, the plasma insulin concentration varied from 0.19 to 0.29 milliunits per milliliter. The authors suggest that the difference in clinical picture presented by these two groups of patients precludes the assumption that the difference is one of severity of the same process.

The effect of exogenous HGF on these same two types of diabetic patients has been investigated by Pincus (181). Five of six patients with labile diabetes showed a rise in blood sugar significantly greater than the rise elicited in either normal subjects or stable diabetic patients. The author suggested that if the labile diabetic is deficient in HGF secretion, "a certain amount of glycogen deposited

in the liver is not used as adequately as in the normal individual." HGF presumably helps mobilize this liver glycogen and in this sense assists the action of insulin by making available to it the hepatic glycogen stores.

The possibility that von Gierke's disease might be due to a deficiency of HGF is a tempting hypothesis. Patients suffering from this malady have been shown to have adequate liver glycogen stores yet their blood sugar levels are abnormally low, indicating that they are unable to mobilize hepatic carbohydrate reserves. However preliminary clinical tests administering HGF to these patients have not proved successful (182).

To conclude this brief review of clinical investigations on HGF, it may be mentioned that McQuarrie et al. have reported a tendency to hypoglycemia in patients with congenital absence of pancreatic alpha cells (183).

It may be convenient at this stage to summarize the more important and better studied properties of HGF discovered up to the present time:

(1) probably a protein

(2) alkali- and cysteine-resistant

(3) solubility properties similar to insulin

(4) stimulates liver glycogenolysis <u>in vivo</u> and <u>in vitro</u>

(5) stimulates the transformation of inactive to active phosphorylase in liver

(6) hyperglycemic

(7) probably hyperketonemic

(8) probably formed by the alpha cells of the islets of Langerhans

(9) apparently physiologically elaborated under the stimulus of hypoglycemia.

As mentioned in the introduction, one of the important aspects in the investigation of a substance such as HGF, is the elucidation of its rôle in human physiology. The writer has approached this problem by searching for HGF in the urine of normal human subjects. It is necessary, therefore, to proceed to review the pertinent literature.

In the course of investigations that led to the discovery that the anterior hypophysis has diabetogenic properties (184), Houssay et al. studied the effect of urinary extracts on the blood sugar of diabetic toads (185). The urinary extract was prepared by shaking the specimen with kaolin in the cold; separating the kaolin by centrifugation and washing it with water. The insoluble material was extracted with 66% alcohol; the ethanol solution separated by centrifugation and concentrated by evaporation. The activity of the concentrate was tested by injecting it into hypophysectomized-depancreatized toads and determining the blood sugar 12 hours after the injection. The blood sugar level was compared to the level found in control animals which were not injected. The urine of 22 normal subjects increased the blood sugar of the test animals by 20 mg% or more in 25% of the tests, while urine from diabetics caused a similar degree of hyperglycemia in 70% of cases. The effect was variable from day to day and a certain degree of overlapping between the two groups was found. Since the animals were hypophysectomized and depancreatized it must be concluded that the action was not mediated by either one of these glands.

In the same year, 1933, Dingemanse and Kober investigated the action of urinary gonadotrophin on the blood sugar concentration of rabbits (186). They found that the intravenous injection of a purified preparation of gonadotrophin derived from pregnancy urine did not raise the blood sugar, even in doses as high as 1000 rat oestrus units. Crude preparations however increased the blood sugar level by 25 to 58 mg%. Following heat treatment the gonadotrophic activity was destroyed but the hyperglycemic effect persisted. Hyperglycemia reached a peak 1½ hours after injection and in general the increase did not exceed 40 mg%.

The problem was independently investigated by Mather, Katzman and Doisy (187), who extracted the urine of normal men with the aid of either benzoic acid or tungstic acid and brucine. Either extract was shown to produce hyperglycemia when injected into rabbits, but the tungstic acid preparations gave a more consistent response. It was found that the activity was lost on standing in the cold or following treatment with acid, alkali or heat. The active material was soluble in water and in aqueous solutions of ethanol, methanol or acetone; however, it was insoluble in ether, absolute ethanol, chloroform or strong acetone solutions. The doses used in these tests were extremely high, corresponding to 1.6 to 14.5 liters of urine. The material was administered subcutaneously to rabbits and hyperglycemia reached a maximum value 3 to 6 hours later with values of 180 to 310 mg%. It is of interest that previously fasted animals did not respond to the treatment, indicating that the hyperglycemia was dependent on adequate carbohydrate stores.

Davis, Hinsey and Markee investigated the possibility that the hyperglycemic action of urinary extracts could be due to constituents other than prolan (188). They observed hyperglycemia in rabbits following the administration of crude prolan, untreated pregnancy urine, urine from a normal man and from a 2-year old male infant. However, the effect did not appear after the intravenous administration of 50-100 units of highly purified prolan. No rise in blood sugar followed the intravenous administration of solutions of sodium chloride (2.5 mg/kg), urea (62.5 mg/kg) or creatinine (2.5 mg/kg). Hyperglycemia was observed following the administration of uric acid (1.5 mg/kg), sodium urate (4.5-5.0 mg/kg), and sodium hippurate (3.5 mg/kg), with rises in the blood sugar level of 20 to 30 mg%. The effect due to either these latter substances or man's urine did not appear after destruction of the adrenal medulla by electric cautery; or after treating the animals with 500 units of theelin. Boiling for 30 minutes destroyed the activity of either urate or normal man's urine. From these observations it was concluded that the hyperglycemic action of urinary extracts is not associated with the gonadotrophic activity present, but is probably due to urates, hippurates and possibly other substances. The action appears to be mediated by the adrenal medulla, a fact which might mean that the effect is non-specific.

In a series of publications that appeared between 1934 and 1936, Harrow and his coworkers investigated both the hyperglycemic and the hyperketonemic properties of urinary extracts (189,192). In their preliminary experiments their extract was prepared essentially in the manner described by Funk (193,194), who had previously demonstrated

the presence of a hyperketonuric substance in urine. Harrow concluded that the product of this preparation is the same as Doisy's extract referred to above, and he called it the "fat-metabolism hormone". The material isolated by this procedure elicits hyperglycemia and hyperketonemia when injected into rabbits in doses ranging from the equivalent of 1.8 to 3 liters of urine. The time at which the maximum blood sugar level was reached, ranged between the first and the sixth hour after the injection, and did not seem to be related to the dose given. No relationship is apparent between the dose administered and the degree of increase in blood sugar, which varied between 207 and 19% of the initial level; this is also true of the maximum rise in blood acetone, which ranged from 75 to 283% of the initial value and occurred within the first two hours following the injection. Other metabolic changes observed were a decrease in carbon dioxide combining power and a raise in blood lactate.

In the course of this work the authors modified the extraction procedure on several occasions. The method summarized below appears to have been the most satisfactory one they found to prepare the crude urinary extract. Three liters of acidified urine was treated with benzoic acid dissolved in alcohol; the precipitate formed was separated by filtration and resuspended in 95% ethanol. The insoluble material was reextracted with alcohol and the residue suspended in normal ammonium hydroxide. The mixture was made alkaline to phenolphthalein, centrifuged and the supernatant made up to 10 ml. before injection. Further purification by dialysis, alcohol precipitation and barium acetate treatment produced a more potent but unstable fraction. After assuming that the urinary substance that induces hyperglycemia is of

pituitary origin, Harrow et al. concluded that it is involved in carbohydrate metabolism, primarily, and in fat metabolism, secondarily (189).

The hyperglycemic activity of the urine of normal human subjects and diabetic patients was tested in dogs and rabbits by Werch and Altshuler (195). The method of preparation of the extracts was similar to the one described by Houssay et al. (185). The authors found a blood sugar raising substance in the urine of severe diabetics and to a lesser degree in the urine of mild diabetic patients. In normal urine they reported little or no hyperglycemic activity. The active material was found to be absorbable by kaolin or charcoal, soluble in water and 60% ethanol, insoluble in fat solvents, heat labile and not filtrable through a Berkefeld filter. It was concluded that probably the substance was not a protein because it gave a negative biuret test, although it could be precipitated by ammonium sulphate and was not dialyzable. The maximal increase in blood sugar was found with extracts prepared from the urine of uncontrolled diabetics receiving insulin. Table 9 presents the range of the average blood sugar increases one hour after injection for all the experiments reported. The authors do not mention what procedure was used to determine the blood sugar, but changes as small as the ones reported for normal subjects are within the experimental error of some methods.

TABLE 9

Type of Patients	No. Patients	Blood sugar increases * mg%
Severe diabetics receiving insulin	2	61 - 63
Mild diabetics receiving insulin	4	44 - 46
Diabetics controlled by diet alone	2	17 - 19
Normal subjects	3	5 - 12

* The writer has pooled the dog and rabbit experiments in this table because the results were similar.

The possibility must be considered that the hyperglycemic activity demonstrated in the urine of patients from the first two groups could have come from the insulin administered. It must be remembered that at the time this work was done (1937) most commercial insulin preparations were contaminated with HGF.

Werch et al. further observed that increasing the dose of extract elicited an almost proportionally higher blood sugar response; however, no attempt was made to develop a quantitative assay. Boiling the extract for one hour destroyed the activity, the increase in blood sugar one hour after the injection being only 8 mg%.

The dose used in these experiments corresponded to about 3.5 hours of urine excretion. It appears, therefore, less likely that the effect elicited would be a non-specific toxic one than in the experiments of Mather et al. (187) or Harrow et al. (190) who used doses corresponding to several liters of urine.

It is surprising to find that none of the investigators who studied the hyperglycemic activity of urinary preparations appear to have been aware of the contemporary experiments of Bürger et al. Some workers like Harrow and Werch suggest that their preparations derive their activity from a pituitary factor that Anselmino and Hoffman had concentrated at about that time (196,197).

It is difficult to evaluate the full significance of these early studies, for, as mentioned above, the doses used by some workers were staggeringly high. Besides, no satisfactory bio-assay was designed and this makes it impossible to establish rigorous quantitative relationships between the various preparations. One gathers

the impression that some of the experiments were designed to satisfy a preconceived theory, and that the early confirmatory results were not critically dealt with in an effort to test the theory.

Although the methods of preparation were not very elaborate and in general somewhat similar, the results were sometimes discordant. Houssay et al. (185), for instance, reported hyperglycemic activity in the urine of 25% of the normal subjects and in 70% of the diabetics tested by their sensitive technique. Werch et al., on the other hand, present convincing evidence that all their assays of normal urine were negative; while none of their specimens from diabetics failed to raise the blood sugar concentration of their test animals. This discrepancy is disquieting since both these groups of workers prepared their respective extracts following essentially the same procedure.

In 1948 Meduna and Vaichulis reported that the urine of schizophrenics contains a greater amount of a hyperglycemic substance than the urine from normal individuals (198). Two methods of preparation are presented: one based on absorption by kaolin in acid medium followed by extraction with weak ammonium hydroxide; the other procedure consisted in precipitating by the addition of acid and drying the precipitate in an air current at room temperature. These crude extracts were tested by intravenously injecting the extract derived from a 24-hour specimen into rabbits weighing 2 kg. Blood samples were drawn 1,2,3 and 4 hours later for glucose determination. Thirty tests were carried out on schizophrenic patients and 21 students and nurses served as controls.

After the administration of extracts derived from the urine of schizophrenics, the blood sugar level of the rabbits reached a maximal value at 2 hours and the extracts from the control urines produced maximal changes one hour after the injection. A graph is presented in which the per cent increase in blood sugar is plotted against time; when the results are expressed in this manner, the difference between the two groups is very marked. However, the individual determinations for any one time interval show a very wide variation as indicated by the Standard Deviations included. The greatest difference between the two groups was found two hours after the injection, and the values are shown below:

	Average Blood Sugar % Rise
Normal subjects	23 ± 29.5*
Schizophrenics	94 ± 39.9

*Standard Deviation.

It is unfortunate that the authors chose to express their results as per cent increase over the pre-injection concentration of blood sugar instead of presenting both the initial and final glucose levels. This would have allowed the reader to better appreciate how consistent the effect was; for it is well known that in rabbits the resting blood sugar concentration is very variable. No attempt was made to analyze the results statistically, which would have helped in the interpretation of their findings, particularly in view of the wide variability found. The present writer has calculated the

probability that the means presented above are different and has found them to be significantly so.

From these observations the authors proceeded to conclude that the increased amount of hyperglycemic fator found in the urine of schizophrenics is related to an anti-insulin factor previously demonstrated in the blood of these patients (199,200). They claimed that schizophrenic patients can be classified into two groups, one of which shows evidence of disturbances of carbohydrate metabolism, including insulin resistance, a pseudodiabetic reaction to the Exton-Rose test and the presence of "anti-insulinic factor in the urine", while the patients of the second group show none of these.

These findings have been confirmed by Walker and Mayer-Gross (201) who prepared extracts from the urine of psychotic patients following Meduna's procedure. These authors present evidence that the crude urinary preparations contain two hyperglycemic agents: a weaker, more quickly-acting one consisting of urates; and a stronger, more slowly-acting factor which is associated with the amino-acid content of the extract and might be a protein. No results were presented regarding the hyperglycemic activity of the urine of normal human subjects. However, the relationship between the uric acid and amino-nitrogen content of the extracts, on the one hand, and the magnitude of the blood sugar rise on the other, suggests that it is the slower-acting but more potent factor, which is increased in the urine of psychotic patients. In this regard it is interesting to note that Meduna found that four hours after the injection into rabbits of extracts derived from normal urine, the blood sugar was back to the preinjection level

in 18 out of 21 subjects tested; while in the case of the schizophrenic patients the average blood sugar level at four hours was 61% above the initial value.

From the reports reviewed above, it appears that a number of investigators have found a hyperglycemic substance in the urine of normal human subjects, although some workers like Werch and Altshuler failed to do so. The urinary excretion of a hyperglycemic agent has been reported elevated in diabetics (185,195) and mental patients (198,201). Although early reports associated the hyperglycemic activity of urine with gonadotrophic substance (186,187), later reports showed that highly purified gonadotrophic preparations did not raise the blood sugar (188). It has further been shown that the intravenous administration to rabbits of a solution containing salts of uric acid produces hyperglycemia (188,201). Walker et al. however, presented evidence showing that in the urine of mental patients there is a factor, other than uric acid, which contributes to raise the blood sugar of test animals (201).

The question arises: is this urinary hyperglycemic substance related to the Pancreatic Hyperglycemic Glycogenolytic Factor? For if both these agents are one and the same, then a link can be established between the experimental studies on HGF reviewed above, and human physiology, and this could provide an approach to the investigation of the rôle that HGF may play in diseases of metabolism. The writer has attempted to answer this question by studying the effect of urinary extracts on the blood sugar of test animals, and on liver glycogenolysis <u>in vitro</u>, in the light of present knowledge concerning the physiological properties of HGF.

C. Experimental Work

1. Methods and Materials

(a) Glucose Determination

Glucose determinations on either blood or incubation fluids were carried out by the method of Folin and Malmros (202). In the part of this work which deals with urinary extracts the more specific method of Nelson (203) was used. Although Nelson claims that the use of Folin-Wu tubes is not necessary, the writer found that when determining small amounts of glucose the use of these tubes was essential to obtain reproducible results.

(b) Glycogen Determination

The method of Good, Kramer and Somogyi (204) was used to separate and hydrolyse glycogen. The glucose was then determined by Nelson's procedure using glucose standards that contained hydrochloric acid in the same concentration as the unknowns. All glycogen values are presented in terms of glucose.

(c) Nitrogen Determination

Nitrogen determinations were carried out by the micro-modification of Johnson's method (205) suggested by Umbreit, Burris and Stauffer (206).

(d) Uric Acid Determination

Uric acid was analyzed by the method of Brown (207).

(e) Manometric Procedures

The determination of oxygen uptake and of aerobic and anaerobic acid formation, was done by the Warburg technique (206). The simultaneous determination of oxygen uptake, acid formation and

respiratory quotient was carried out with the aid of Dixon-Keilin flasks and Summerson differential manometers (206).

(f) Buffer Solutions:

Phosphate-buffered saline was prepared by mixing 100 ml. of 0.9% sodium chloride with 20 ml. of 0.11 M phosphate buffer (pH 7.4).

Krebs-Ringer-Bicarbonate solution was prepared in the conventional manner without the addition of glucose.

(g) Crude Pancreatic Extract:

The starting material used to purify HGF was the mother liquor from the first isoelectric precipitation of insulin* in the extraction process of Romans, Scott and Fisher (153). This material had been found by Stewart (139) to be rich in hyperglycemic and glycogenolytic activity and for this reason it was chosen as a source of HGF. The liquor, which will be referred to as FIP, was shipped from Toronto as a clear liquid but in transit a variable amount of precipitate formed. FIP derived from either beef or hog glands was used in the course of this investigation,and the purer preparations of HGF reported below, were obtained with hog extract. According to the manufacturer each liter of FIP corresponded to 125 lbs. of fresh pancreas. The pH on arrival invariably was 5.15 and the nitrogen content of cattle FIP was found to be 1.62 mg./ml.

*Supplied by kindness of Drs. D.A. Scott and A.M. Fisher, Connaught Laboratories, Toronto.

2. Procedure to Assay Pancreatic Glycogenolytic Preparations

Before attempting to purify FIP to any extent, it was necessary to develop an assay procedure that would permit the evaluation of potency of the various fractions. Considerable effort and time was spent in this aspect of the work, and this considerably delayed the original program, which was to attempt the purification of HGF present in the supernatant of the first isoelectric precipitation of insulin.

When the present project was begun, two methods of assay of HGF had been previously described. Stewart's method (138) determined the hyperglycemic activity of HGF preparations by injecting them intraperitoneally into rats and measuring the blood sugar 30 minutes later. In this procedure, each dose tested was injected into two animals and their response compared to that of untreated control rats. The linearity of the Dose-Response curve obtained by this method appears to be satisfactory, but the slope of the curve is low, which would indicate that the procedure is not very sensitive. In spite of this disadvantage, the method appears to have proved useful because the agreement in response between similarly treated animals was quite close. Another disadvantage of the method is that it requires relatively large amounts of the material tested, particularly since the low sensitivity of the test object would impose a multiple-dose assay.

For these reasons it was thought that the assay procedure of Sutherland and Cori (154) should be preferred. This method of assay determines the potency of glycogenolytic preparations by incubating liver slices in phosphate-buffered saline in vitro in the presence or absence of the material under test, and comparing the amount of glucose

released into the medium under these two conditions. The general principle of the procedure appeared in the literature in 1948 (154,155), at about the time the present work was begun. Further technical detail was published the following year by the same group of workers (161). However, in these studies no detailed information was given as to what variability might be expected from replicate determinations using slices from the same liver. In their 1949 paper Sutherland et al. (161) state, "Examples will be given for comparative assays of the same preparation of H-G factor on liver slices of different animals. The agreement was satisfactory with the exception of two cases in which the variation was 25 to 30 per cent". This suggests that the agreement from animal to animal was better than 25%, and presumably also the agreement between duplicate slices within each assay; however no information is given on this latter point. The present writer is of the opinion that such uniformity in response, from a body constituent as variable as liver glycogen, can only be expected from a pure strain of animals, housed under rigidly controlled conditions. He has found more recently that the liver glycogen of New Zealand white rabbits, when determined after homogenizing the tissue in phosphate-buffered saline, can vary from 2 to 12%, even when the animals are of the same sex, approximately the same body weight and kept on the same dietary regime fed ad libitum. It attests to the difficulty of the procedure that a year and a half after their first publication, Sutherland et al. were still adding technical directions on how to perform the assay (161). In a personal communication Dr. Sutherland very kindly supplied still more detailed directions, which proved very useful to the writer.

Before describing the assay procedure used in this work, it is necessary to discuss some factors that were found to influence the variability of response. One such factor was the time required to prepare and weigh the slices. According to Sutherland (161), this could be as long as 40 minutes provided the tissue had been properly cooled first. He also recommended that the preparation be carried out by two operators: one to slice the tissue and the other to weigh the slices. To circumvent the necessity of a helper, the slices were kept on a piece of hard filter paper (Whatman No.50) moistened with cold saline; the paper was placed in a Petri dish which in turn was kept in an ice bath. Although this arrangement considerably decreased the variability of response of replicate slices, it was not until Dr. Marcel Chaput joined the project, and assisted in the preparation of the slices, that reasonably consistent results were obtained. In connection with the effect of low temperature, it should be mentioned that repeated pouring of cold saline over the tissue, during the slicing operation, appeared to affect the results favorably, and therefore this minor modification was introduced in the routine assays.

In an effort to maintain the tissue at a low temperature as long as possible, free-hand slicing between pre-cooled glass plates was preferred to the Stadie-Riggs microtome. The thickness of the slices was checked by weighing slices that had been cut to measure 1 square centimeter in area, and from the weight, the thickness of the slice was calculated. After a little practice it was possible to cut slices 0.3 to 0.5 mm. thick quite consistently. However, it became apparent after a time that an ordinary razor blade could not be used to cut more than 2 or 3 slices per edge, and considerable tearing of the tissue was observed if a longer series was prepared with the same blade.

The lack of an adequate device to shake the incubation vessels at a constant temperature, required the improvisation of the following arrangement: slices were incubated in open test-tubes stood in a constant temperature bath, and the incubation fluid agitated by blowing oxygen through it. By means of a manifold as many as 12 tubes could be aerated simultaneously. This arrangement proved useful, but not infrequently uncontrollable changes in oxygen pressure resulted in violent bubbling and the loss of fluid from some of the tubes. When a long-awaited mechanical shaker arrived, the aerating procedure was gladly abandoned.

The initial choice of test animal was an unfortunate one, for the liver glycogen stores of the rat are lower than those of the rabbit. Swensson (208) has reported that rabbit liver contains about 9 per cent glycogen, while the rat liver usually contains about half that concentration. The lower liver glycogen content of the rat may have accounted for the irresponsive animals occasionally encountered. In the latter part of this work the rabbit was adopted as test animal, a modification that reduced the number of irresponsive animals and increased the sensitivity of the test.

The procedure used in most of the assays reported below will now be described. To facilitate the comparison of different fractions derived from the same starting material, and to obviate the necessity of doing nitrogen determinations on inactive intermediate fractions, whenever possible, the various sister fractions were diluted in like manner and the assay performed on the same volume of each solution. As a rule the fractions were dialyzed before assaying to remove

extraneous crystalloids that could interfere with the test, and immediately before testing the pH was adjusted to values within 6.5 and 8. The pH of the incubating fluid containing the solution under test was measured with the glass electrode, and was invariably found to be 7.4 ± 0.1. When solid fractions were assayed, they were dissolved directly in phosphate-buffered saline.

New Zealand white rabbits weighing from 2 to 5 kg. were anaesthesized by an intravenous injection of Nembutal and the liver immediately removed, cut in large pieces and cooled in isotonic saline in an ice bath. After cooling for 15 minutes one operator cut slices, either free hand or with the aid of a Stadie-Riggs microtome, while the other weighed them on a torsion balance to the nearest 0.2 mg. Ten slices could be prepared in about 30 minutes, and during this time they were kept on cold, moistened filter paper as described above. The tissue was transferred to large test tubes containing 2 ml. of phosphate-buffered saline and 0.2 ml. or less of the solution tested. The tubes were shaken at a rate of 120 oscillations per minute in a constant temperature water bath at 37°C for one hour. At the end of the incubation, aliquots of the fluid were removed for glucose determination. It was found possible to prepare another 10 slices while the first series was incubating, and this was usually done. Table 10 presents the results found in an assay designed to test the potency of fraction 1.6, the preparation of which will be described later.

It is apparent that the effect is graded but the variability within each dose level made it difficult to establish the potency of the preparation. It was possible, however, to compare two preparations

by repeated assays using more slices at a single dose level. In spite of this shortcoming the assay proved valuable, particularly because the more potent preparations were fortunately obtained through clean separations of activity.

TABLE 10

Dose µg./ml.	Response* Series 1	Series 2
400	110	96
	117	149
200	84	--
	170	--
100	73	--
	57	--
10	24	--
	19	--
1	--	0
	--	-5

*Percent increase in glucose output of individual slices over untreated slices.

The word "series" on the table refers to the first and second group of slices prepared for this assay.

In retrospect the writer feels that possibly too much was expected from the testing procedure, and that statistical analysis of the results plus adequate designing of the experiments, would have undoubtedly increased the value of the method of assay. Recently Audy and Kerly have published results of quantitative assays of glycogenolytic activity conducted by a similar technique (209). A single preparation was tested on 8 rabbits, and in each experiment approximately four slices were treated with a standard preparation, and four others were incubated in the presence of the same dose of an unknown preparation. Table 11 presents the results found by these authors

with the first three rabbits tested in a series of 8. The remaining animals are omitted since these three suffice to exemplify the variability of the values obtained.

TABLE 11

Rabbit No.	Response* Standard	Unknown
15	95,83,64,64	42,40,27,21
17	63,73,77,84	12,30,38,19
20	138,151,152 217,282	30,39,71

*Percent increase in glucose output of individual slices over untreated slices.

In spite of the great variability found with different animals and with different slices from the same tissue, it was concluded that the method "gives an approximate estimate of activity". However for greater accuracy the authors propose a multiple-dose method requiring the aid of intricate statistics.

The assay procedure used in the present work was a reliable enough index of activity to guide the exploratory purification work presented below. As a rule 2 or even 3 rabbits were used to compare the potency of two or more preparations. The result of each assay in the following section represents the average of at least one pair of slices. When the ratio of duplicate determinations was greater than 1.15 or smaller than 0.85, the results were discarded.

3. Preliminary Extraction Experiments:

FIP was concentrated by freezing. The starting material was frozen with a mixture of dry ice and ethanol until the volume of unfrozen solution was approximately one tenth of the original volume. This

concentrated fluid was collected by centrifugation, and fractionated further.

200 cc. FIP was transferred to a 500-ml. beaker which was then immersed in a larger beaker containing dry ice and ethanol. The solution was allowed to freeze until a small volume of unfrozen fluid remained; this unfrozen core was poured off and measured (12 ml.); the pH was 5.1. The concentrate was centrifuged and a precipitate separated, and resuspended in 20 ml. distilled water; this will be referred to as fraction 1. Enough water was added to make the volume of the supernatant fluid up to 20 ml.; this is fraction 2. After allowing the ice to melt at room temperature a precipitate settled out; it was collected by centrifugation and resuspended in 20 ml. water (fraction 3). These preparations were assayed by the addition of 0.2 ml. of each solution to 4 ml. of phosphate-buffered saline per incubation tube. It can be seen from the results given in Table 12 that the activity appeared to be about equally distributed among all three fractions. For this reason this line of approach was not pursued further.

TABLE 12

Fraction	Dose ml.	Response*
1	0.2	40
2	0.2	60
3	0.2	35
FIP	0.2	14

*Percent increase in glucose output over untreated slices.

Rendering the starting material alkaline (pH 9) before freezing dissolved most of the solid in suspension, however the activity again

appeared distributed over the various fractions. Attempts to concentrate the activity by freezing followed by alcohol precipitation proved unsuccessful.

4. Fractionation with trichloroacetic acid:

The addition of trichloroacetic acid (TCA) to FIP until a concentration of 5% was reached resulted in the precipitation of very active material. Although the supernatant retained some of the activity it was decided to fractionate by these means with TCA concentrations ranging from 0 to 5%, since this procedure appeared to offer a selective means of concentrating the active material.

200 ml. FIP was chilled in an ice bath to 3-6°C. A 10% solution of TCA was then added in five 40 ml. portions from a dropping funnel with constant stirring. Each portion was added over a 20 minute period and stirring continued for another five minutes. During this time a precipitate formed and was separated by centrifugation. Each precipitate was dissolved in 15 ml. of 0.1 N sodium hydroxide, the pH adjusted to 6.7 by the addition of dilute HCl and water added to a final volume of 20 ml. The solutions were then dialyzed for 24 hours against distilled water in the cold. Five fractions were prepared in this manner at the following TCA concentrations expressed in per cent: 1.6, 2.8, 3.7, 4.4, 5.0. Fraction 1.6 was found to be by far the most active one and therefore it was decided to fractionate between 0 and 1.6% TCA concentrations, and also to collect all insoluble material at 5% TCA.

The procedure used in this fractionation was essentially the same as described above, except that fractions were separated not at prefixed TCA levels but at those concentrations at which precipitation

occurred. As each precipitate formed the mixture was placed in the cold until precipitation appeared to be complete. The precipitates were collected, dissolved, diluted and dialyzed, as described above, and 0.2 ml. aliquots used for assay. The fractions will be referred to by the TCA concentrations at which each precipitate formed. The results of this assay are presented in Table 13, from which it can be seen that fraction 1.6 is the most active one, containing almost one half the total activity found in the four preparations.

TABLE 13

Fraction	Response*	% Total Activity Found
0.4	3.1	25
1.1	1.8	14
1.6	5.6	44
5.0	2.1	17
	12.6	100

*Mg. glucose per gm. fresh tissue, minus control value.

In the course of this preparation it was observed that after separating fraction 1.1, further addition of TCA produced no change until the concentration was approximately 1.3%. At this point the solution became progressively more cloudy as the acid was added, until a heavy precipitate flocculated. It was decided therefore to separate the material precipitable at a TCA concentration of 1.3% before proceeding to precipitate fraction 1.6. This was done as follows: to 200 ml. FIP was added 30 ml. 10% TCA over a 10 minute period, as previously described, and stirring continued for 3 hours. The precipitate formed was separated by centrifugation, taken up in 20 ml. of water, washed with ether and dialyzed against cold distilled

water for 48 hours with repeated changes. This will be referred to as fraction 1.3 (f-1.3). To the supernatant (218 ml.) was added 8 ml. 10% TCA over a period of 5 minutes and stirring continued for 3 hours. After centrifugation the precipitate was taken up in 20 ml. of water, washed with ether and dialyzed against cold distilled water for 48 hours with repeated changes. This is fraction 1.6 (f-1.6).

Nitrogen determinations on these fractions yielded the following values:

Fraction	mg.N/ml.
FIP	1.62
f-1.3	2.02
f-1.6	0.82
Supernatant from f-1.6	0.92

As a rule 85 to 95% of the nitrogen in FIP was accounted for, the values presented above being within this range.

Table 14 presents the results of assays conducted on fractions prepared in this manner. Assays 1 to 3 were conducted on the same preparations and Assay 4 on fractions prepared at a later date.

TABLE 14

Assay No.	Fraction	Dose ml.	Dose mg.N	Response*
1	1.3	0.2	0.40	210
	1.6	0.2	0.16	240
2	1.3	0.2	0.40	63
	1.6	0.2	0.16	51
3	1.3	0.2	0.40	208
	1.6	0.2	0.16	178
4	1.3	0.2	---	56
	1.6	0.2	---	54

*Percent increase in glucose output over untreated slices.

It appears that both these preparations are equally active per unit of volume, but since the nitrogen content of fraction 1.3 is considerably higher than that of fraction 1.6, it was decided to use this latter preparation as starting material for future purifications.

It was of interest to know whether fraction 1.6 (f-1.6) contained insulin, and this was tested by injecting a diluted and neutralized solution into rats and determining the blood sugar concentration before and 30 minutes after the injection as recommended by Hrubetz (210). Table 15 presents the results of this experiment, from which it was concluded that f-1.6 was contaminated with insulin.

TABLE 15

Rat	Dose mg.N	Decrease in Blood Sugar mg.%
1	0.25	33
2	0.25	25
3	0.50	71
4	0.50	58

5. Purification of f-1.6

When the pH of fraction 1.6 was adjusted to 6.6, a precipitate formed. This precipitate was collected by centrifugation, dissolved in dilute alkali, the pH adjusted to 6.1 and enough distilled water added to make the volume up to the initial volume of f-1.6; this is fraction 4. The supernatant fluid after precipitating at pH 6.6 is fraction 5, which was found to retain all the activity as shown by the representative assay presented under Assay 1, Table 6.

The glycogenolytic activity was found to be best preserved in an acid medium at low temperature, but even under these conditions, the

various fractions were found to become progressively less active. It was decided to dry a partially purified preparation in an effort to preserve the activity. Two procedures for drying were employed: lyophilization, and blowing air with a fan on a dialyzing bag containing the solution in question. An aliquot of fraction 4 was dialyzed against running tap water for 18 hours at 7°C; the dialyzed solution is fraction 6. Two 5 ml. aliquots of fraction 6 were dried by the procedures described above. The aliquot that was air-dried yielded 44.4 mg. of a brittle yellowish material; while 56.2 mg. were collected after lyophilization. These are fractions 7 and 8 respectively, and the results of two different assays conducted on them appear under Assay 2, Table 16. These results indicate that no appreciable amount of activity was lost by dialyzing, but that approximately one half the potency disappeared when the solution was either air-dried or lyophilized. Nevertheless dry preparations were extensively used in the course of this work because of their general conveniency and stability. It may be of interest to mention that freezing previously dialyzed commercial insulin did not markedly decrease its glycogenolytic activity.

Purifying f-1.6, as follows, yielded a more active preparation on a weight basis. After removing the material precipitable at a concentration of 1.3% TCA, f-1.6 was separated by centrifugation and dissolved in 15 ml. of distilled water (pH 2.5). Sodium hydroxide (0.1N) was added dropwise until a precipitate formed at pH 4.7. The insoluble material was separated by centrifugation and resuspended in 15 ml. of water (fraction 9). More dilute alkali was added to the supernatant

until the pH reached a value of 8.3; no precipitate appeared and since the activity was destroyed in alkaline solutions the pH was not raised beyond this value. A 5 ml. aliquot of the supernatant was treated

TABLE 16

Assay	Fraction	Dose		Response[1]
		ml.	mg.[2]	
1	4	0.2	--	120
	5	0.2	--	8
2	4	0.2	--	100,100
	6	0.2	0.5[3]	82,102
	7	0.2	0.5	46,42
	8	0.2	0.5	45,50

(1) Percent increase in glucose output over untreated slices
(2) mg/ml. incubating fluid
(3) estimated

with dilute hydrochloric acid to adjust the pH to 6.4, it was then dialyzed in the cold against distilled water for 24 hours with repeated changes. After drying at room temperature in an air current, 3.0 mg. of solid were obtained (fraction 10). Table 17 presents the results of assays performed on these fractions.

TABLE 17

Fraction	Dose ml.	Response*
1.6	0.4	31,39
9	0.4	22,-7
10	0.4	58,54,33,47

*Percent increase in glucose output over untreated slices

It appears from the figures in this table that fraction 9 was inactive. Further, fraction 10 was about 1½ times as active as f-1.6,

since the dilution and dosage of both preparations were identical. On a weight basis f-10 was approximately 10 times as active as fractions 7 or 8, since a comparable response was obtained with only 55 µg. per ml. of incubating fluid.

The most active fraction obtained in the course of this work was prepared as shown by the flow sheet on Figure 2. Fraction 1.6 was prepared as previously described. The trichloroacetic acid was then removed by extracting with ether and the aqueous solution dialyzed against distilled water in the cold for 2½ days with repeated changes. The solution had now a pH of 6 and on prolonged standing at room temperature (about 8 hours) a precipitate formed. After removing the solid by centrifugation, further standing at room temperature brought down a second crop of precipitate. The supernatant is referred to as fraction 11 and the combined precipitates as fraction 12. The first precipitate was taken up in a volume of distilled water equal to the volume of fraction 1.6 from which it had precipitated. The second precipitate was dissolved in this solution (pH 5). Addition of dilute HCl to f-11 until pH 4.3 was reached produced a precipitate, f-14, which was separated by centrifugation. Fraction 13, the supernatant at pH 4.3, was immersed in a boiling water bath for 5 minutes and allowed to stand in the cold for 1½ hours. At the end of this time a precipitate had formed; it was separated by centrifugation and labelled f-16. The supernatant will be referred to as f-15. Table 18 summarizes the results of assays performed on the active fractions 11,13 and 15; fractions 12,14 and 16 were found to be inactive. Doses given on the table are comparable, since all fractions were diluted to the same volume.

Figure 2

Method of Obtaining f-15

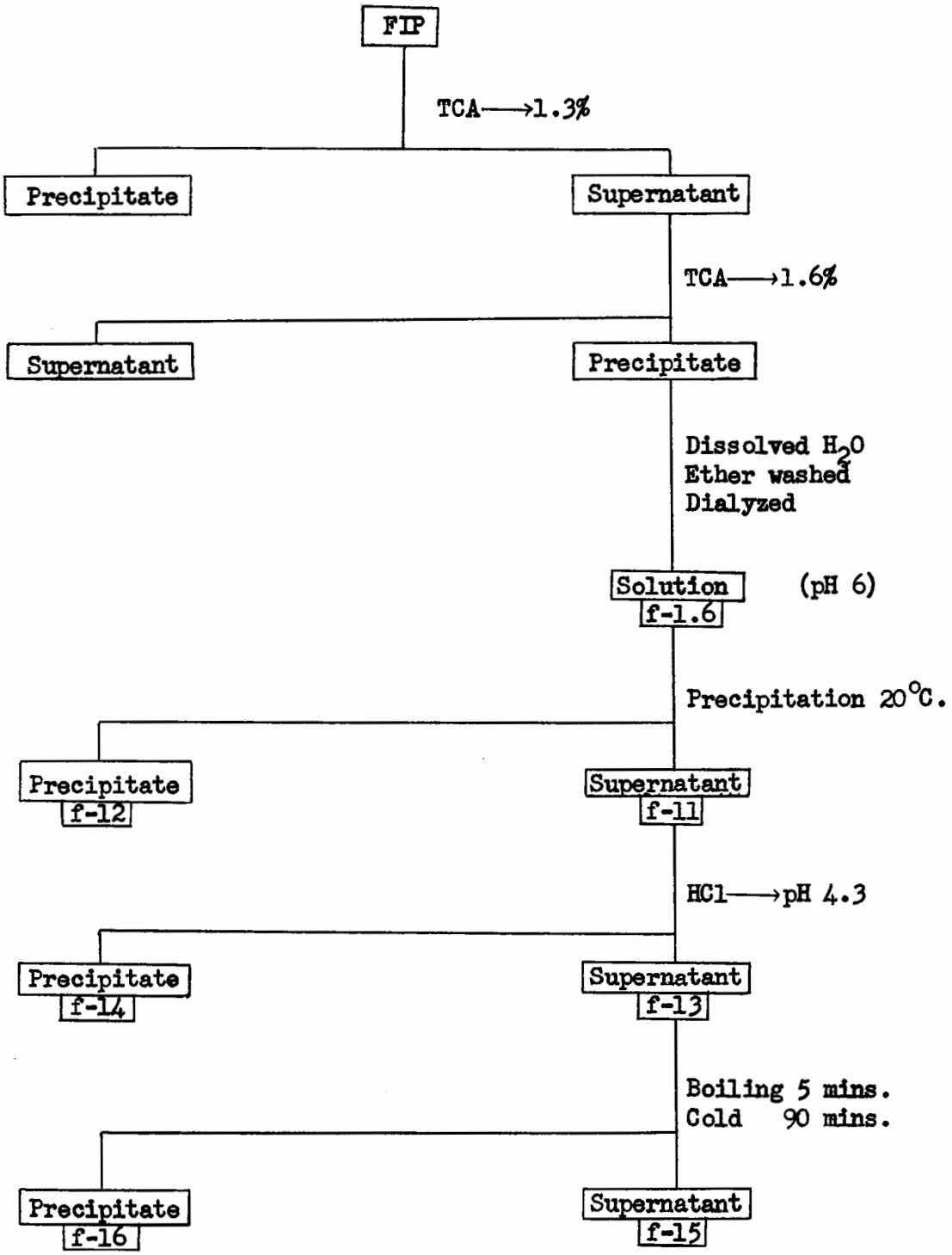

TABLE 18

Assay No.	Fraction	Dose ml.	Response*
1	1.6	0.1	81
	11	0.1	78
2	13	0.1	41
3	13	0.1	31
	15	0.1	24

*Percent increase in glucose output over untreated slices.

During this procedure clean separations of activity were obtained. It may be seen from the table that there was little loss of activity in passing from f-1.6 to f-11, and from f-13 to f-15. Unfortunately it is not possible to compare fractions f-11 and f-13 as analytical data are not available.

Fraction 15 was found to be active at a dose level of 7.1 µg. of nitrogen per ml. of incubating fluid and represents the purest fraction prepared in the course of this study.

6. Miscellaneous Experiments

Since some of the fractions that were prepared markedly increased the glucose output of liver slices, it was thought of interest to study what effect they might have on metabolism as reflected by oxygen uptake and acid formation in vitro. Table 19 presents the results of experiments on the effect of fractions 1.6 and 1.3 on the oxygen consumption of liver slices incubated in phosphate-buffered saline, using the Warburg technique. It appears from these results that neither f-1.3 nor f-1.6 markedly affected the oxygen uptake of liver slices, although both fractions induced an increase in the rate of release of glucose into the medium.

TABLE 19

Experiment	Species	Treatment	Dose µg./ml.	Oxygen uptake*	Average
1	Rat	f-1.3	400	160,138	149
		f-1.6	400	164,152	158
		Control	--	152,149	150
2	Rabbit	f-1.6	400	49,51,61	54
		Control	--	50,41,40	44

* $\mu l.O_2$ consumed /100 mg. fresh tissue /60 minutes.

The *in vitro* effects of a glycogenolytic preparation were studied with the aid of Dixon-Keilin vessels and differential Summerson manometers, an apparatus which permits the simultaneous determination of the oxygen utilized by the tissue, the acid formed under aerobic conditions and the Respiratory Quotient. Representative results of these experiments appear in Table 20.

TABLE 20

Experiment	Species	Treatment	Dose µg./ml.	Oxygen Uptake (1)	Acid Formed (2)	R.Q.
1*	Rat	f-1.6	200	126	42	0.60
		Control	--	101	23	0.67
		"	--	102	20	0.68
2	Rat	f-1.6	200	224	67	0.51
		Control	--	131	57	0.44
3*	Rat	f-1.6	200	137	13	0.73
		"	200	134	6.5	0.72
		Control	--	153	4	0.62
4	Rabbit	f-1.6	100	71	84	0.49
		Control	--	41	43	0.44
5	Rabbit	f-1.6	100	121	85	0.52
		"	100	90	89	0.44
		Control	--	122	44	0.83
		"	--	80	46	0.69

(1) $\mu l.O_2$ consumed /100 mg. fresh tissue /90 minutes
(2) Acid expressed as $\mu l.CO_2$ evolved /100 mg. fresh tissue /90 minutes
* "Oxygen Uptake" and "Acid Formed" values for 60 minutes.

It can be seen from Table 20 that when liver slices were incubated

in Ringer-Krebs-Bicarbonate medium, the presence of f-1.6 sometimes induced an increase in the oxygen uptake (Experiments 2 and 4); however, the effect was not constant and in some experiments either there was a small increase of doubtful significance or no change at all. The action on the respiratory quotient, in general, appears to be too small to be significant and it is not a consistent effect, since in some experiments the R.Q. was slightly raised and in others somewhat depressed. The only consistent effect found was a marked increase in acid formation, usually of the order of 100%. In only one experiment (#2) was a small increase found, and it is perhaps significant that in this instance treatment with f-1.6 markedly increased the oxygen uptake.

The action on aerobic acid formation was also found in simple Warburg experiments in which the composite pressure changes were plotted against time. Experiment 1 in Table 21 shows that under these conditions, the gas evolution of rabbit liver slices incubated for one hour in bicarbonate medium was markedly increased by f-1.6, the difference being 71% higher than the gas evolution of untreated slices. The glucose output during the incubation (after subtracting the glucose released during the preincubation period) was increased by 83% under the influence of f-1.6.

Further investigation showed that the increase in acid formation found with f-1.6 was independent of any effect this preparation may have had on the oxygen consumption of the tissue, since when incubated anaerobically the slices formed more acid in the presence of f-1.6. These results are illustrated by Experiments 2 and 3 in Table 21.

TABLE 21

Experiment	Gas Phase	Treatment	Dose µg./ml.	Dose U./ml.	Gas Evolution*	Average
1	O_2	f-1.6	100	--	52,58,49	53
		control	--	--	26,29,37	31
2	N_2	f-1.6	100	--	72,72	72
		Insulin	--	1	28,21	24
		Insulin + f-1.6	-- 100	1 --	71,92	81
		control	--	--	33	33
3	N_2	f-1.6	100	--	105,136	120
		Insulin	--	1	54,43	51
		Insulin + f-1.6	-- 100	1 --	137,129	133
		control	--	--	66,84	75

* Experiment 1: composite pressure changes per 100 mg. fresh tissue per hour, assuming rise in pressure entirely due to acid formation.
Experiments 2 and 3: µl.CO_2 evolved /100 mg.fresh tissue /120 minutes.

From the same experiments it can also be seen that a commercial insulin preparation known to be contaminated with HGF did not stimulate acid formation, but rather depressed it by about 30%, when added to the medium in a concentration of 1 unit per milliliter. When rabbit liver slices were treated with both insulin and f-1.6, the acid formation was increased to the level attained by f-1.6 alone. In view of these experiments it appears possible that the effect of f-1.6 on acid formation may be due to substance(s) other than HGF.

No further studies were carried out on the purification of HGF. Interest was then focused on searching for this material in the urine of normal human subjects.

7. Preparation and Properties of Crude Urinary Concentrates

Specimens of urine were collected from normal human subjects during a 24-hour period and kept in the cold until the collection was completed. The untreated urine was dialyzed against cold running tap water for about 18 to 24 hours, and dialysis continued for 12 to 24 hours against cold distilled water with repeated changes. This solution was concentrated by blowing air around the suspended dialysing bags at room temperature. The concentrates from the various bags were pooled, the bags washed with small portions of distilled water, and the resulting solution (about 100 ml.) lyophilized. The weight of solid material obtained varied approximately from 300 to 700 mg. in the seventeen concentrates prepared by this procedure.

When injected into rabbits these preparations raised the blood sugar concentration. Figure 3 shows two representative experiments where rabbits received intravenously 20 and 40 mg. of urinary concentrate per kilogram of body weight dissolved in 4 ml. of phosphate-buffered saline. As a rule the blood sugar concentration was increased by about 70 to 100 mg.% and the maximum value was reached 30 to 60 minutes after the injection. Table 22 presents the maximal blood sugar levels observed following the administration of different urinary concentrates. The figures under "Minutes" indicate the time after injection at which the maximum response was reached.

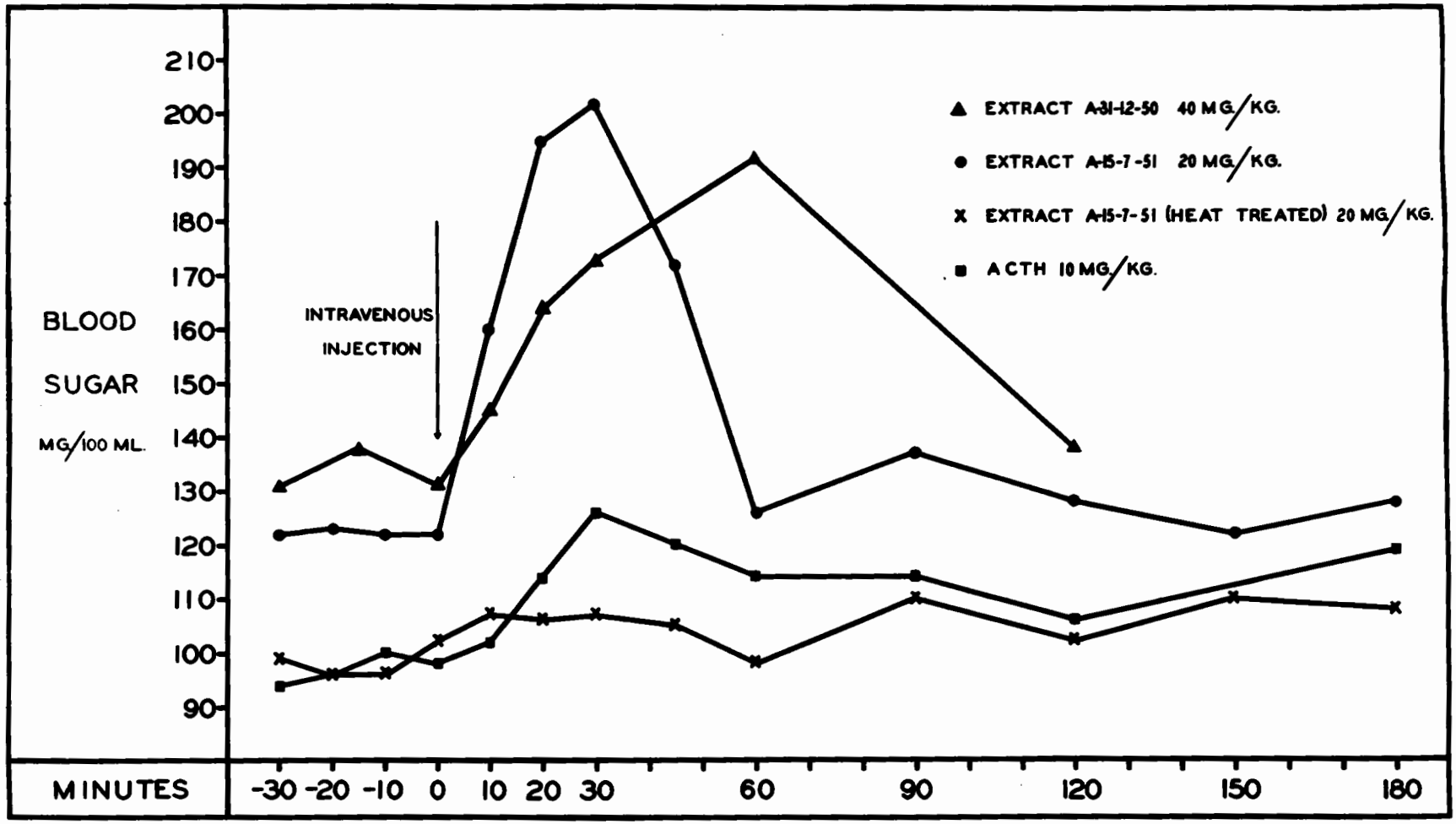

Figure 3 - Blood glucose changes following intravenous injection of urinary concentrates and ACTH in normal rabbits. In each case the material injected was dissolved in 4 ml. of phosphate-buffered isotonic saline (pH 7.4).

TABLE 22

Experiment	Concentrate	Dose mg./kg.	Maximum Blood Sugar Concentration	
			mg%	Minutes
1	A-9-12-50	42	203	60
2	A-9-12-50	29	172	30
3	A-31-12-50	37	220	120
4	A-31-12-50	42	192	60
5	A-3-4-51	43	169	30
6	A-3-4-51	43	271	90
7	A-15-7-51	20	206	30

Examination of Table 22 brings out two points of interest. The doses used to elicit marked hyperglycemia are very much lower than any previously reported in the literature. In Experiment 1, for instance, the total amount of material injected was equivalent to 240 ml. of urine corresponding to less than 3½ hours of excretion. Secondly the time at which hyperglycemia was most marked varied considerably, even for the same preparation, and was possibly dependent on the liver glycogen concentration at the time of the experiment. In general the hyperglycemic peaks reached at 30 minutes tended to be lower than those reached at a later time.

The possibility that this effect might not be a specific one was investigated by administering gelatin and ACTH in the same manner. Gelatin at a dose level of 40 mg./kg. produced no change in the blood sugar. ACTH when given in doses corresponding to 10 or 20 mg. of Armour's Standard LA-1-A raised the blood sugar by 25 to 30 mg% only, as shown in Figure 3. Furthermore, after heating an aqueous solution of an active preparation at pH 5.5-6 in a boiling water bath for one hour, it failed to affect the blood sugar level; this is shown in Figure 3. For these reasons it was concluded that the hyperglycemic

activity of the urinary concentrates was not a non-specific one.

The concentrates increased the rate of release of glucose by rabbit liver slices incubated in vitro. Following the assay procedure described above, it was found that the amount of glucose released into the medium was roughly proportional to the concentration of urinary material. From Figure 4 it can be seen that a dose of 600 µg./ml. elicited approximately maximal response, while 150 µg./ml. was about half as effective. These amounts of concentrate correspond to approximately 1.4 and 0.35 minutes of urinary excretion.

The values in Figure 4 were obtained from a series of 5 rabbits, and each dot on the graph represents one pair of slices that agreed within 15% of each other.

There appears to be no uric acid in these preparations, since repeated attempts to demonstrate its presence failed. The concentration of the solution tested was sufficiently high to allow the detection of uric acid, even if it constituted only 1% of the weight of concentrate. The nitrogen content of the crude concentrates was found to be approximately 7%.

Following alkali treatment the material did not completely lose its ability to stimulate the output of glucose by liver slices in vitro. The alkali treatment was carried out as follows: 10 mg. of concentrate A-9-12-50 was dissolved in 25 ml. of 0.08 N sodium hydroxide and shaken at 37°C for 3½ hours. The solution was then neutralized, dialyzed overnight against distilled water in the cold, and lyophilized. In order to assay it, the dry material was dissolved in 20 ml. of phosphate-buffered saline, and therefore each milliliter of solution contained the equivalent of 500 µg. of original concentrate. The results of this Assay appear in Table 24, under fraction D-27-12-50.

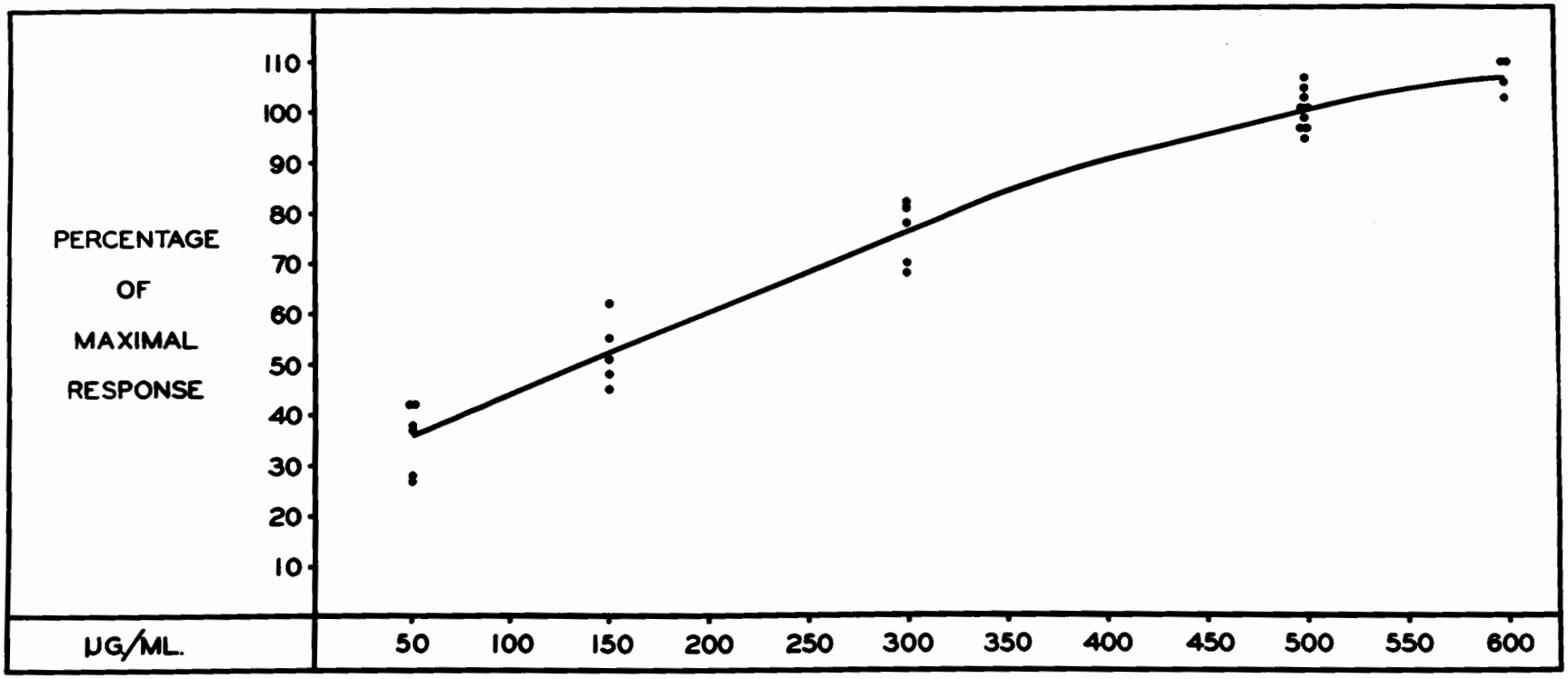

Figure 4 - Changes in glucose content of the medium following incubation of rabbit liver slices with various amounts of urinary concentrate dissolved in 1.2 ml. of phosphate-buffered isotonic saline. The results are expressed as percentage of maximal response.

The active material was found to be precipitable by ammonium sulphate. Four milliliters of saturated ammonium sulphate (SAS) was added dropwise and with stirring to 4 ml. of phosphate-buffered saline containing 100 mg. of concentrate A-9-12-50. After standing overnight, a precipitate was separated by centrifugation, dissolved in water, dialyzed free of sulphate ion and lyophilized. This will be referred to as fraction A-27-12-50. To the supernatant, solid ammonium sulphate was added until an excess remained undissolved. The precipitate and the supernatant were separated by centrifugation, dialyzed and lyophilized. They will be referred to respectively as B-27-12-50 and C-27-12-50. The weight and nitrogen content of each of these fractions appear in Table 23 and the assay of their potency in Table 24.

TABLE 23

Fraction	Treatment	mg.	% Nitrogen
A-27-12-50	Precipitate ½ SAS	55	9.5
B-27-12-50	Precipitate SAS	9.8	9.5
C-27-12-50	Soluble in SAS	3.4	18.0
A-9-12-50	--	--	7.0

The combined weight of the ammonium sulphate fractions was 68.2 mg.; therefore about one third of the original 100 mg. of crude concentrate are not accounted for, and presumably were eliminated in the process of dialysis. The nitrogen content of each of these fractions was higher than that of the starting material.

It can be seen from Table 24 that the material precipitated at half saturation with ammonium sulphate is as active as the original

TABLE 24

Fraction	Treatment	Dose µg./ml.	Response (1)
A-9-12-50	--	500	6.2
A-27-12-50	Precipitate ½ SAS	500	6.2
B-27-12-50	Precipitate SAS	500	3.4
C-27-12-50	Soluble SAS	100	0.2
D-27-12-50	Alkali	(500)*	3.6

(1) Increase in glucose output as mg./gm. tissue (average of two slices), after subtracting output of untreated controls.

* Corresponds to 500 µg. of concentrate before alkali treatment.

concentrate on a weight basis, while the precipitate at 100% saturation retains about half the activity. Little can be said about the activity of the material soluble in saturated ammonium sulphate, since it was assayed at a dose lower than the other fractions. However this material appears to be inactive, since by weight it was tested at 1/5 the dose at which the starting material (A-9-12-52) responded maximally, and yet it did not produce a significant increase in glucose output.

The properties of the crude urinary concentrate that have been investigated may be summarized as follows:

The material:

(1) is hyperglycemic when given to rabbits

(2) increases the glucose output of liver slices <u>in vitro</u>

(3) is non-dialysable

(4) is at least partially inactivated by heat

(5) is resistant to mild alkali treatment

(6) is partially precipitable by ammonium sulphate

(7) is free of uric acid

(8) contains about 7% nitrogen.

These properties are not incompatible with the interpretation that the crude urinary concentrate owes its hyperglycemic action to the presence of HGF. However this information is far too limited to draw any definite conclusions on the question. Later experiments proved that these two hyperglycemic agents are not the same, neither do they appear to have the same mechanism of action.

The crude urinary concentrate described above appeared to be very impure, as evidenced by the fact that a maximal response could be elicited only with 500-600 µg./ml. incubating fluid. It was therefore decided to attempt extraction of the urine, in an effort to produce a purer preparation.

8. Further Purification and Investigation of Urinary Hyperglycemic Factor.

It was found that the addition of 2 volumes of 95% ethanol to native urine, or urine rendered acid or alkaline, precipitated the active material. The supernatant retained relatively little activity.

A specimen of urine was separated into two aliquots of equal volume. One aliquot was dialyzed and lyophilized as described above; this will be referred to as fraction A-30-9-51 (250 mg.). The second aliquot was filtered through Whatman No.2 paper, rendered acid (pH 5) by the addition of 10% HCl, and 2 volumes of 95% ethanol added dropwise and with stirring. The mixture was allowed to stand at 4°C overnight. The precipitate formed was separated by centrifugation and dissolved in water, dialyzed as described above and lyophilized. This is fraction B-30-9-51 (150 mg.). The supernatant from the alcohol precipitation was similarly dialyzed and lyophilized, but only a very small amount of black residue remained after drying, and it was discarded. The results

of assays conducted on these fractions are shown on Table 25 under Assays 1 and 2, from which it can be see that on a weight basis, the alcohol-precipitated material is more active. It can also be seen from Assay 2 that the activity of 50 µg. of the alcohol precipitated material is about the same as the activity of 400 µg. of the crude preparation. From this it was concluded that with the aid of alcohol approximately an eight-fold purification could be effected.

TABLE 25

Assay	Fraction	Treatment	Dose µg./ ml.	Response*
1	A-30-9-51	Dialyzed Lyophilized	150	1.1, 1.1
	B-30-9-51	EtOH insoluble	150	2.2, 2.4, 2.1
2	A-30-9-51	Dialyzed Lyophilized	400	4.7, 5.9
	B-30-9-51	EtOH insoluble	150	5.3, 5.9
		" "	50	5.4, 4.0
		" "	10	1.2, 1.6
3	A-2-11-51	Dialyzed Lyophilized	50	2.0
	B-2-11-51	EtOH insoluble Water soluble	50	3.6, 4.8
	C-2-11-51	EtOH insoluble Water soluble	50	3.2, 3.4
4	A-12-2-52	Dialyzed Lyophilized	200	20.9, 21.2
	A-28-2-52	Insoluble SAS	200	18.7, 17.9
	B-28-2-52	Soluble SAS	200	16.3, 16.8

* Increase in glucose output as mg./gm. fresh tissue (values of individual slices), after subtracting output of untreated controls.

In the course of this preparation it was observed that the alcohol insoluble material did not completely redissolve in water. Separation

at this stage did not prove advantageous for both soluble and insoluble substances were about equally active. Assay 3 in Table 25 presents these findings.

Attempts were made to purify the lyophilized alcohol precipitate by means of salt fractionation, but fractions both soluble and insoluble in saturated ammonium sulphate (SAS) proved to be equally active, as shown by Assay 4, Table 25. Fifteen milligrams of starting material (A-12-2-52) was dissolved in 15 ml. of water and solid ammonium sulphate added with stirring at room temperature until excess solid remained undissolved. Stirring was continued for 1 hour before separating the precipitate by centrifugation. The precipitate was taken up in water and both this solution and the supernatant fluid dialyzed against running tap water for 28 hours, and against distilled water in the cold for 16 hours. At the end of this time the dialysates were free of sulphate and the solutions were lyophilized. The supernatant (B-28-2-52) yielded 2.1 mg. and the precipitate 2.8 mg. (A-28-2-52).

The presence of uric acid in the alcohol-insoluble, dialyzed and lyophilized material was investigated. Concentrated solutions of this substance (100 mg.%) did not contain detectable amounts of uric acid. Further investigation revealed that the alcohol-insoluble material was contaminated with this urinary constituent. However, following dialysis overnight in the cold with four changes of dialysate, no uric acid was detected in the impermeate. It appeared therefore that following alcohol treatment, uric acid could be removed even by relatively short periods of dialysis, and that the preparations tested were free of uric acid. Uric acid added to the incubation medium in concentrations up to 100 µg./ml. did not stimulate glycogenolysis by liver homogenates, which responded normally to APU.

The amount of material obtained by the alcohol precipitations was usually between 50 and 150 mg. per twenty-four hour specimen, and the nitrogen content was approximately 5%. The preparations were negative to the Hopkins-Cole test either in solution or in dry form, and the xanthroproteic test was also negative. A strongly positive reaction was observed in the Millon-Nasse test and a faint blue coloration with biuret reagent.

In an effort to standardize the testing procedure, it was decided to investigate the tissue glycogen changes during the incubation of liver slices, with the view in mind that glycogen changes might be more consistent than the changes in glucose secretion. It was found that under the stimulus of the alcohol precipitate derived from urine (APU), the increase in medium glucose over the output of untreated slices, was sometimes not accompanied by an additional decrease in liver glycogen; this is shown by Experiments 1 to 3 in Table 26. The total glycogen disappearance, however, exceeded by far the total glucose secreted, and therefore there was no direct evidence of increased carbohydrate synthesis. In some instances (Experiments 8-11, Table 26) glycogen disappearance due to APU was considerably smaller than the increase in glucose secretion by the slice.

Only in four experiments out of 11, (Experiments 4 to 7), did sufficient additional glycogen disappear to account for the rise in glucose output. The positive differences in Table 26 represent an apparent increase in carbohydrate, and this additional glucose presumably could be derived either from gluconeogenesis or from diminished utilization of carbohydrate by the tissue. The ability of the tissue

TABLE 26

Experiment	1	2	3	4	5	6	7	8	9	10	11
Glycogen Decrease (1)	-0.6	-0.7	-0.8	-14.0	-7.8	-4.3	-3.1	-3.1	-1.8	-1.9	-9.4
Glucose Increase (2)	3.3	6.2	3.8	4.0	2.9	2.5	3.2	4.4	4.4	4.2	12.4
Difference	+2.7	+5.5	+3.0	-10.0	-4.9	-1.8	+0.1	+1.3	+2.6	+2.3	+3.0

(1) mg. glycogen (as glucose)/gm. tissue. Net decrease after subtracting decrease of untreated slices.
(2) mg. glucose in medium/gm. tissue. Net increase after subtracting increase of untreated slices.

to increase its glucose output and preserve its glycogen stores either wholly or in part, could not be correlated with the initial glycogen content, for it was found in tissue in which the glycogen at the beginning of incubation ranged between 3 and 9%.

It was then considered that possibly the pancreatic and urinary hyperglycemic factors were two different substances, perhaps acting through different mechanisms. Sutherland et al. (154) reported that HGF did not increase the glucose output of liver homogenates, and the present writer's attempts to demonstrate this effect with crude pancreatic extracts confirmed Dr. Sutherland's findings. It was decided therefore to determine what effect, if any, the urinary substance had on glycogenolysis and glucose secretion by liver homogenates. A homogenate and slices were prepared from the same liver and incubated in the presence of either HGF* or APU; untreated control tissue preparations were also included in these experiments. The doses tested (10 µg./ml. of HGF and 100 µg./ml. of APU) had been previously found to elicit approximately the same glucose output. The results of three such

* Kindly supplied by Eli Lilly Co. of Canada, Toronto.

experiments appear in Table 27. Another experiment is represented graphically in Figure 5.

TABLE 27

Experiment		Slices		Homogenate	
		Pancreatic	Urinary	Pancreatic	Urinary
1	Glycogen Decrease(1)	-9.6	-4.9	-3.8	-61.6
	Glucose Output (2)	7.9	4.5	0.6	19.2
	Difference	-1.7	-0.4	-3.2	-42.4
2	Glycogen Decrease	-7.1	-3.3	-0.6	-64.0
	Glucose Output	6.8	7.1	0.3	20.5
	Difference	-0.3	+3.8	-0.3	-43.5
3	Glycogen Decrease	-4.9	-3.4	-2.5	-68.9
	Glucose Output	2.4	4.6	0.8	19.7
	Difference	-2.5	+1.2	-1.7	-49.2

(1) mg. glycogen (as glucose)/gm. tissue. Net decrease after subtracting decrease of untreated slices.
(2) mg. glucose in medium/gm. tissue. Net increase after subtracting increase of untreated slices.

Examination of Experiments 2 and 3 of Table 27 shows that, although APU appears to induce only limited glycogenolysis in slices, the reason for this is not a deficiency on the part of the tissue, for under the stimulus of HGF greater amounts of glycogen disappeared. In two out of the three experiments presented, the increase in glucose output induced by APU cannot be accounted for by additional glycogen disappearance. The inability of HGF to stimulate the glucose output of liver homogenates was confirmed; APU, on the other hand, induced most vigorous glycogenolysis in homogenates, and under its effect the tissue lost 84 to 91% of the

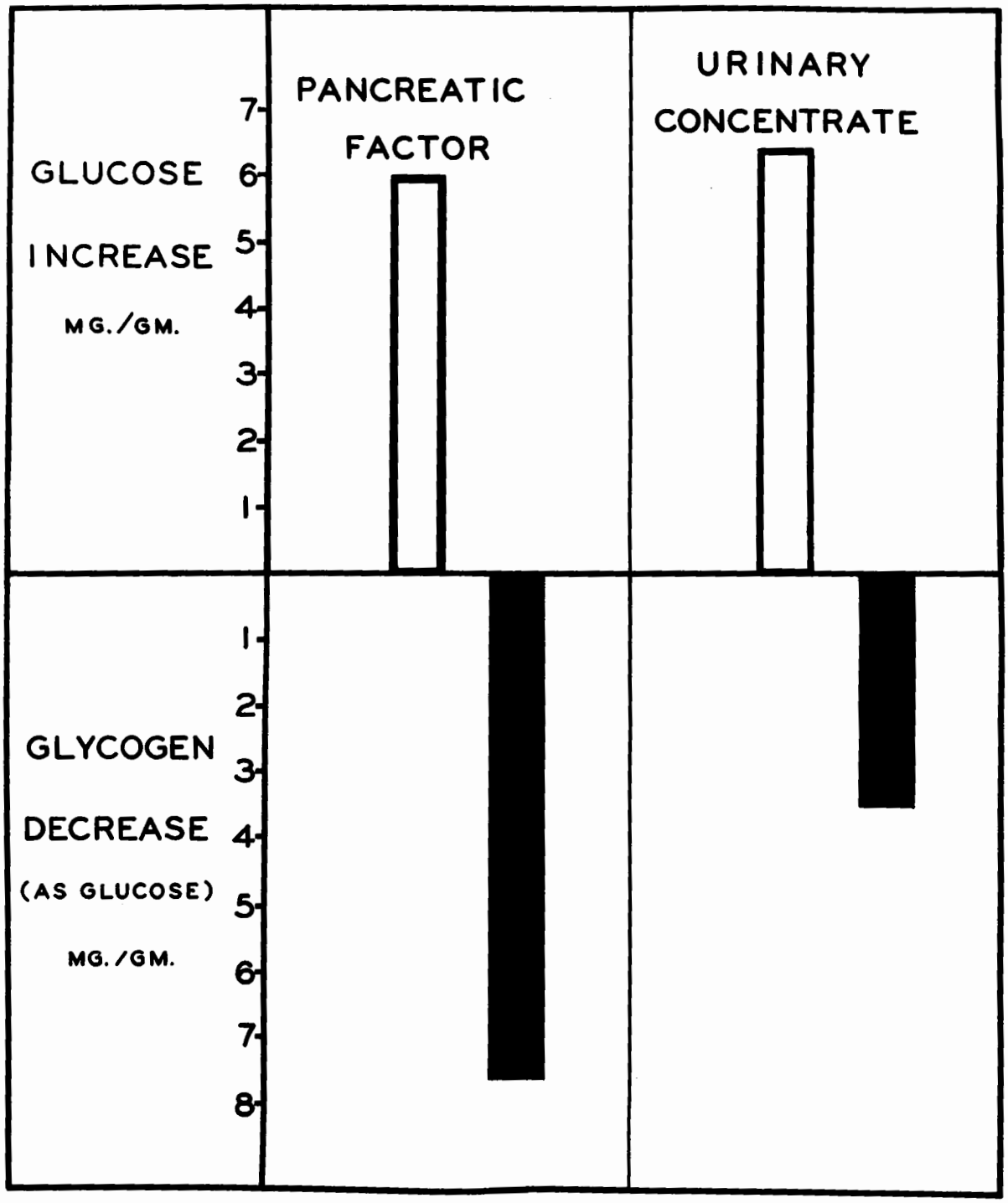

Figure 5 - Medium glucose and tissue glycogen changes after incubation of rabbit liver slices with urinary concentrate and pancreatic factor for 60 minutes. The bars represent the net changes obtained by subtracting the values found for untreated slices from the treated slices.

initial glycogen, as opposed to decreases ranging between 4 and 7% in slices treated in the same manner. These experiments show that, whereas HGF requires intact tissue to stimulate glycogenolysis, APU is not only capable of inducing liver glycogen breakdown in homogenates, but it appears most active when the tissue is in this state. From these observations it was concluded that, in all probability, the glycogenolytic substance found in urine is not HGF.

To elucidate the mechanism of action of APU it was decided to study how it affected the oxygen uptake of liver slices. The results of these experiments appear in Table 28. In Experiment 1 a crude urinary concentrate was incubated with rat liver slices. The second experiment presents the results obtained when rabbit liver slices were incubated in the presence of HGF (Lilly) or APU.

TABLE 28

Experiment	Species	Treatment	Dose µg./ml.	Oxygen Uptake*	Average
1	Rat	A-15-7-51	500	200, 189	195
		"	250	234, 234	234
		Control	--	185,180,195	197
2	Rabbit	B-30-9-51	100	110, 132	121
		HGF	50	126, 125	126
		Control	--	120,117,108	115

* $\mu l.O_2$ consumed /100 mg. fresh tissue /90 minutes.

From these manometric experiments it was concluded that the oxygen consumption of liver slices was not markedly affected by APU.

Calcium in a 0.6 mM concentration has been reported to have a glycogenolytic effect on liver slices (211); therefore it was decided

to investigate the possibility that the very active glycogenolysis elicited by APU in liver homogenates may be due to this ion. Table 29 presents the results found when either slices or homogenate were incubated in the presence of calcium (0.6 mM final concentration, which is equal to 24 µg. of calcium per ml.). The slices were introduced in tubes containing the calcium dissolved in isotonic saline, and the phosphate buffer was added immediately after. The homogenates were prepared in phosphate buffered saline and the calcium, dissolved in saline, added last. It can be seen from Experiment 1 in this table that in agreement with Chaput and Heard, calcium was found to increase the glucose output of liver slices. However, the same calcium concentration had little or no effect on either glycogen breakdown or glucose release by liver homogenates, as shown in Experiment 2.

TABLE 29

Experiment	Tissue Preparation	Treatment	Dose µg./ml.	Glycogen Decrease (1)	Average	Glucose Increase (2)	Average
1	Slices	APU	20	--	--	8.9 7.0 8.9	8.3
		Calcium	24	--	--	4.8 3.6 3.9	4.1
2	Homogenate	APU	200	36.2 36.2	36.2	23.4 22.3	22.8
		"	20	19.9	19.9	13.3 13.2	13.2
		Calcium	24	0.0 -0.3	-0.2	1.4 1.3	1.4

(1) mg. glycogen (as glucose) /gm. tissue. Net decrease after subtracting decrease of untreated slices.

(2) mg. glucose /gm. tissue. Net increase after subtracting increase of untreated slices.

It is apparent from the carbohydrate changes in Experiment 2 that calcium did not stimulate glycogenolysis by liver homogenate, when added in a concentration at which APU elicits marked glycogen breakdown. From these experiments it was concluded that APU does not stimulate glycogenolysis by liver homogenates by virtue of any calcium it may contain, although direct analysis of calcium was not performed.

Following heat treatment APU preparations failed to increase the amount of glucose released by liver homogenates. The preparations were inactivated by heating in a boiling water bath for one hour after dissolving them in phosphate-buffered saline.

In vitro glycogenolysis by rabbit liver homogenates was not affected by a preparation of growth hormone* in doses up to 200 µg./ml. The same tissue preparation responded to APU in the usual manner.

9. Assay of Urinary Glycogenolytic Activity and Daily Excretion by Normal Men

In view of the fact that the urinary extracts were found to be very active glycogenolytic agents when tested on liver homogenates, it was decided to investigate the possibility of using this tissue preparation as test object.

Homogenates prepared from rabbit and rat livers were compared, to determine which species best suited the test procedure. At each dose level 50 mg. of tissue, suspended in 1.2 ml. of phosphate-buffered saline, was incubated at 37°C for one hour, air being the gas phase. From Table 30 it can be seen that rat liver has low responsiveness and that

* Kindly given by Dr. R. D. Stewart.

maximal output is reached with relatively low doses, thus decreasing the useful range of the curve. For these reasons it was decided to perform the assays on rabbit liver suspensions.

TABLE 30

	Glucose Output (mg./g. tissue)				
Dose µg./ml.	2.5	5.0	10.0	75.0	100.0
Rat	1.5	2.4	3.1	2.8	2.9
Rabbit	8.1	13.9	20.0	27.6	28.4

The length of the period of incubation was investigated next. A rabbit liver suspension was incubated as described above, in the presence of 20 and 200 µg./ml. of active material, and the incubation was allowed to proceed for different lengths of time (30, 60 or 90 minutes). The glucose content of the suspension medium was determined, and the results appear in Figure 6. This graph shows that, in an untreated suspension, only about half of the glucose found at the end of the experimental periods was actually formed during the incubation, the remainder having been present at zero time. After 60 minutes of incubation, the tissue treated with the lower dose (20 µg./ml.) was actively releasing glucose, but the tissue incubated in the presence of 200 µg./ml. of APU, showed a decreasing rate of glycogenolysis. At 90 minutes, the higher dose seems to have exhausted the glycogen substrate, while the rate of glycogenolysis induced by the lower dose was in the decrease. The changes after the 30-minute incubation were quite marked, but the difference between the control tissue and the preparation treated with 20 µg./ml. was not very great. It was decided

therefore to perform the assays by incubating the tissue for 60 minutes.

In the course of trial experiments it was observed that the maximal glycogen decrease closely corresponded to the initial concentration of this substance in the tissue. At doses from 100 to 200 µg. of APU/ml. of incubating fluid, close to 100% of the initial glycogen disappeared during a 60-minute incubation period. Figure 7 shows examples of the responses obtained with three different animals having different initial glycogen concentrations. These concentrations are indicated by the figures in parentheses at the extreme right of each curve. It can be seen that in each case, under the influence of a sufficiently high concentration of APU, glycogenolysis reduced the glycogen stores to a value close to zero. The liver glycogen content in these rabbits was found to be exceedingly variable, and frequently undesirably low values were observed. To obviate this difficulty it was decided to pretreat the animals with glucose to raise their carbohydrate stores.

The assay procedure finally adopted was as follows: a rabbit was anaesthesized by an intravenous injection of Nembutal, and this was followed by 20% glucose solution (10 ml./kg. body weight) by the same route over a period of approximately 30 minutes. Two hours later the animal was bled under Nembutal anaesthesia and the liver removed and cooled in ice-cold saline for 15 minutes. A piece of tissue was blotted dry with filter paper, rapidly cut into slivers, weighed on a balance (sensitivity $\pm$ 0.01 gm.) and homogenized in phosphate-buffered saline. Aliquots of this suspension containing 50 mg. of tissue were added to tubes (23 x 150 mm.) containing the material under test dissolved in phosphate-buffered saline, the combined volume being 1.2 ml. Additional tubes were prepared to determine the initial glycogen

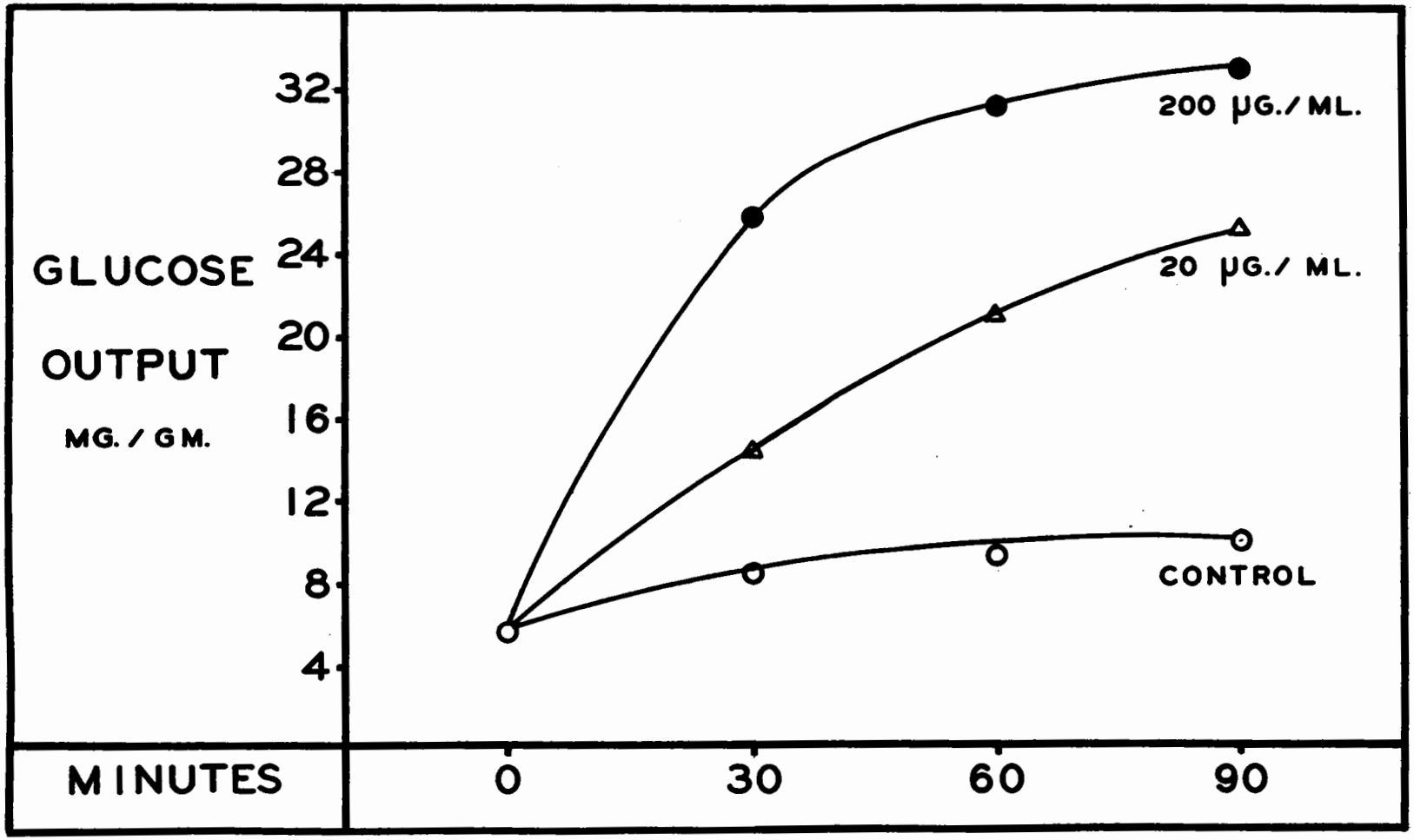

Figure 6 - Glucose output of rabbit liver homogenate in the presence of urinary glycogenolytic material, during different incubation periods.

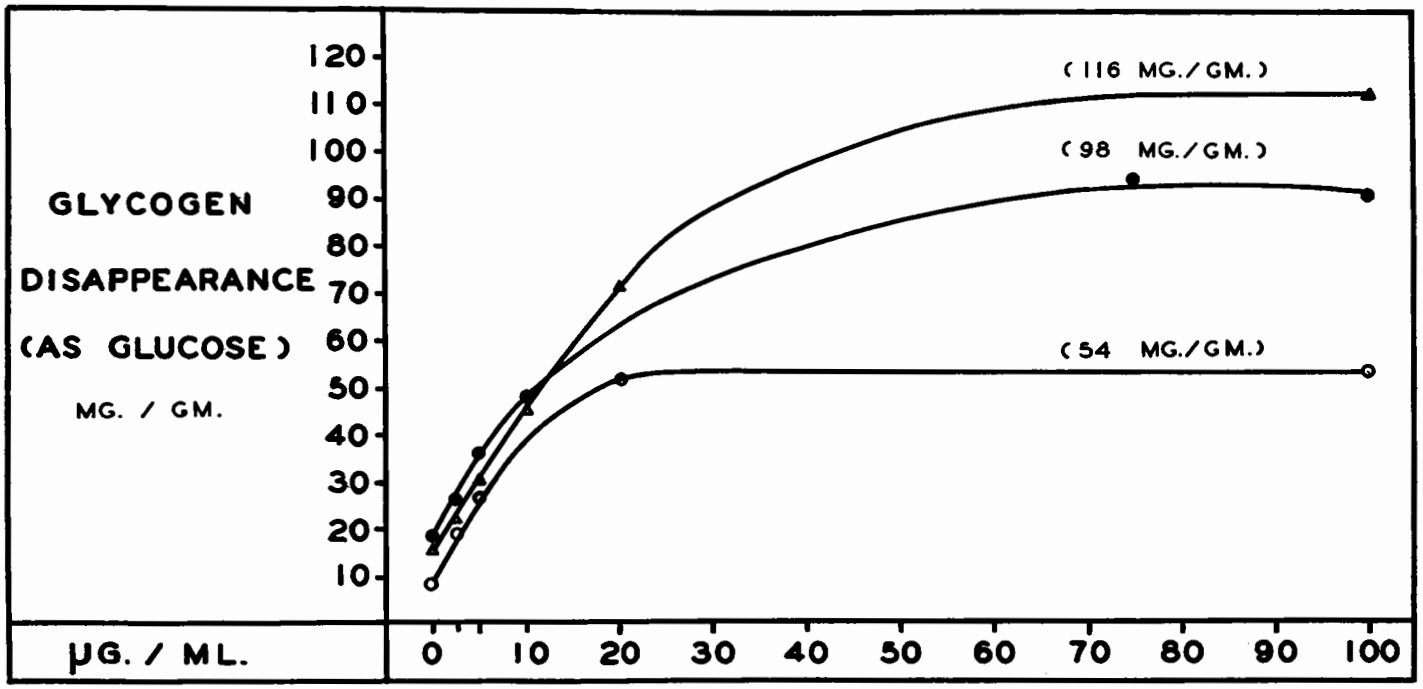

Figure 7 - Glycogen disappearance in rabbit liver homogenates incubated in the presence of different amounts of urinary glycogenolytic material. The figures in parentheses at the extreme right indicate the initial glycogen content of the homogenate.

content of the suspension. The tubes were incubated at 37°C in a constant temperature water bath provided with a shaking device, which was adjusted at 120 oscillations per minute. At the end of one hour the total suspension was treated with 1.2 ml. of 60% potassium hydroxide and heated over a small flame to dissolve the tissue. Glycogen was determined by the method referred to above. The response was calculated by subtracting the amount of glycogen found in each tube from the initial glycogen present at zero time. Figure 8 illustrates a typical assay, and it can be seen that the response is quite linear at dose levels below 15 µg. per ml. With each group of unknown extracts assayed, a series of different concentrations of a reference preparation were tested and the potency of the unknown material read from a curve similar to the one shown in Figure 8. A unit of activity has been tentatively defined as the potency of 1 mg. of the reference substance.

Using the assay procedure described above the daily excretion of glycogenolytic substance has been tested in 24-hour specimens of urine from normal human subjects. A simplified method of extraction was adopted, and it was expected that by rigorously adhering to it, the procedure itself would not introduce any variations.

Twenty-four specimens of urine were collected, so that collection would finish with the first morning specimen of the second day. The urine was refrigerated during the time of collection. The following characteristics of the specimen were noted: volume, colour, appearance and pH; and the sugar, albumin and creatinine content determined. A 10-ml. aliquot of mixed urine was separated and 20 ml. of 95% alcohol

added to it after adjusting the pH to 5 with 10% hydrochloric acid. The mixture was kept in a refrigerator for 4 hours, and was then centrifuged. The supernatant was discarded and the tube containing the precipitate carefully drained and wiped dry of residual fluid. The precipitate was dissolved in a volume of isotonic saline corresponding to 10 minutes of excretion. This volume is calculated as follows: $\frac{V \times 10}{1440}$, where V is the total volume of urine and 1440 is the number of minutes in 24 hours. The solution was dialyzed against isotonic saline in the cold. Dialysis was begun at approximately 4 p.m. and the saline changed at 9 p.m., midnight, 3 a.m., 6 a.m. and 9 a.m. In mid-morning the solution was centrifuged and any insoluble material present was discarded. A 5-ml. aliquot of the supernatant was diluted with an equal volume of saline and 2 ml. of 0.11 M phosphate buffer (pH 7.4) was added. This is dilution A. The phosphate and sodium chloride content of this solution are those of phosphate-buffered saline.

This solution was assayed by mixing a 0.7 ml. aliquot with 0.5 ml. of tissue suspension (containing 50 mg. of liver) in phosphate-buffered saline. The extract was also assayed at half this dose level by diluting an aliquot of dilution A with phosphate-buffered saline and taking a 0.7 ml. aliquot for the test. Each dilution was assayed in duplicate.

Using this procedure of extraction a series of 17 specimens from 6 normal men ranging in age from 23 to 43 years have been assayed for glycogenolytic activity. The daily excretion has been found to vary approximately from 20 to 200 units, all specimens but one contained 40 units or more. This same range of variation was found in 7 specimens

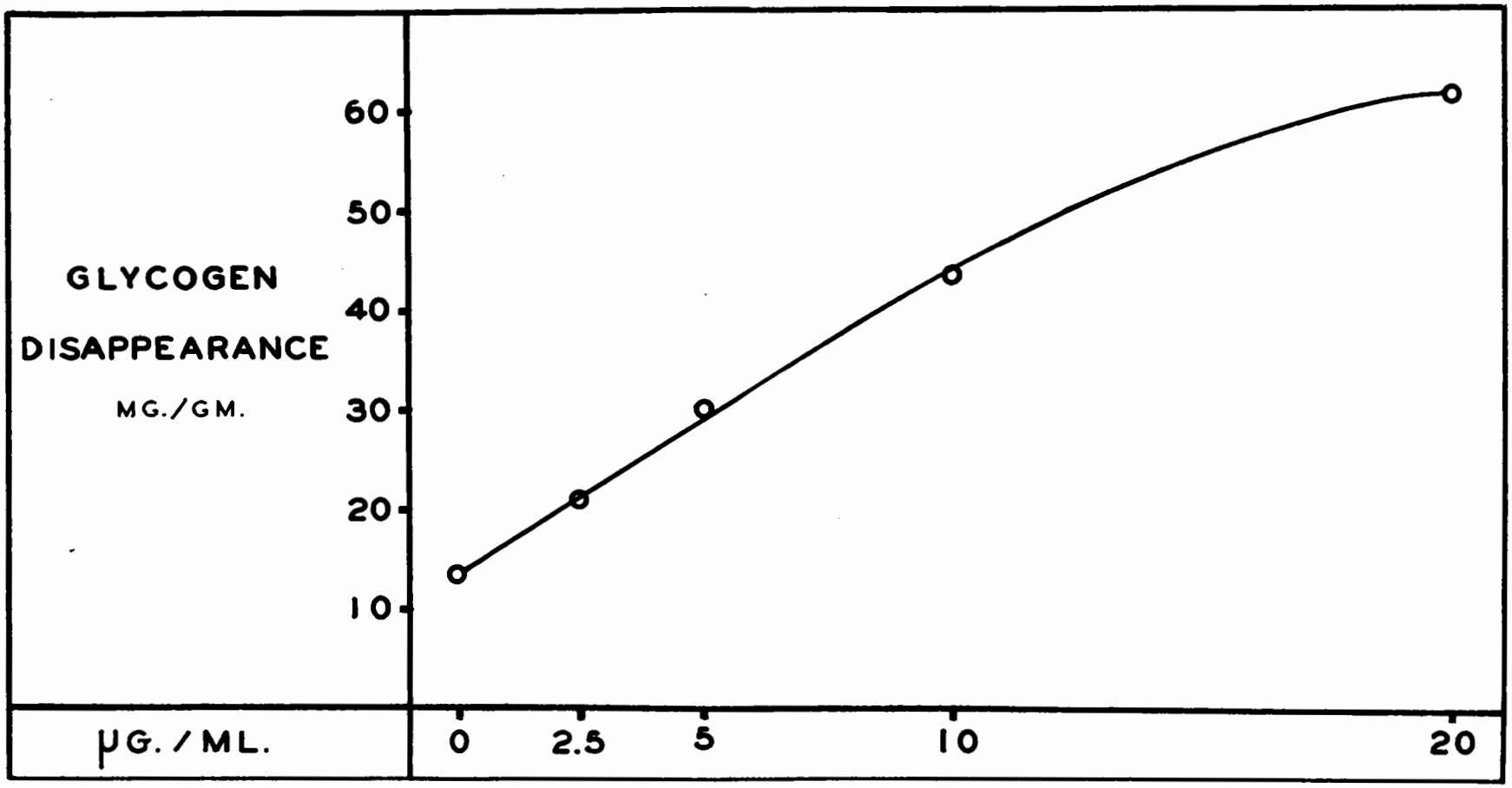

Figure 8 - Dose-Response curve of rabbit liver homogenate incubated in the presence of various concentrations of urinary glycogenolytic material.

from the same individual, collected at different times over a period of 9 months. This series of determinations is not extensive enough to draw any conclusions from it, but it is expected that a larger number of assays might serve to establish the normal rate of excretion of this material. With this knowledge on hand it would then be possible to investigate the rate of excretion in diseases involving derangement of carbohydrate metabolism, and this might help to elucidate what rôle, if any, this substance plays in human pathology.

D. Discussion

The experiments presented in Part II above can be divided into two groups: those related to the study of the glycogenolytic material derived from pancreas; and those directed to elucidate what rôle, if any, this substance plays in the regulation of carbohydrate metabolism in man.

The original project to study pancreatic HGF called for its purification from the supernatant of the first isoelectric precipitation of insulin in the method of Romans, Scott and Fisher (153). It was further planned to study <u>in vitro</u> the biochemical properties of the purified preparation. Only a modest measure of success was attained in this phase of the work, for considerable difficulty was encountered in setting up the assay procedure described by Sutherland et al. (154, 155,161), and as a result the purification work was much delayed. The purest material obtained (fraction 15, p.98) represented a marked concentration and purification of the active material, since an appreciable response was obtained with as little as 7 μg. of nitrogen

per milliliter of incubating fluid. However for purity, this material can hardly be compared to some of Sutherland's preparations which elicited half-maximal glycogenolytic response at dose levels under 1 µg. of protein per milliliter (161). Fractionation with trichloroacetic acid was shown to be an advantageous method of separating HGF from much of the inactive material. This finding was reported at about the same time as Sutherland's communication appeared, showing that TCA in a concentration of 1.5% precipitated part of the HGF found in gastric mucosa. It has been claimed (163) that extracting the "15% sodium chloride precipitate" of crude insulin in the procedure of Romans et al. (153) with 80-90% ethanol, and fractionating the insoluble material with 2.5% TCA, produces a precipitate which is hyperglycemic and insulin-free. Under the conditions of the experiments reported here, it was found that the glycogenolytic material that precipitated from FIP at a concentration of 1.6% was contaminated with insulin.

Thus it can be seen from these studies that HGF could be concentrated and partially purified from FIP, a waste product of insulin preparation, by a procedure different from those commonly used to prepare insulin. The active material was further shown to be stable for many months after dialysis and lyophilization.

The <u>in vitro</u> metabolic studies showed but one consistent and pronounced effect, an increase in acid formation by liver slices incubated under aerobic or anaerobic conditions. The significance of this observation is questionable, since an insulin preparation that had been shown to be contaminated with HGF, did not elicit this effect. This makes it seem likely that the increased acid production is due to some contaminant in the preparations tested.

The search for HGF in the urine of normal human subjects was not successful. A hyperglycemic, glycogenolytic preparation was obtained from urine, but it does not appear to be HGF, though sharing some properties with it. The best evidence of non-identity is the ability of the urinary material to vigorously stimulate glycogenolysis by liver homogenates, a property not shared by HGF. On the positive side, it can be claimed that a naturally-occurring hyperglycemic-glycogenolytic substance has been found in the urine of normal men and women. This substance does not appear to be epinephrine which, like HGF, requires intact cellular structure to elicit glycogenolysis in vitro (166). The urinary preparations were shown to be free of uric acid, a substance previously shown to be hyperglycemic when injected into rabbits (188). Furthermore, uric acid did not promote glycogenolysis by liver homogenates under the conditions used to test APU activity. The addition of calcium also failed to markedly affect glycogenolysis by liver homogenates, and it was concluded, therefore, that APU did not act by virtue of contamination with this ion. The test object used to assay APU also failed to respond to growth hormone, which when highly purified has been shown to be diabetogenic in the dog (212), and in the rat when combined with ACTH (213). The identity of the active material in APU remains unknown, but a few possibilities seem to have been ruled out.

Besides being precipitable by the addition of alcohol to acidified urine, crude preparations of the active substance derived from urine have been found to be non-dialysable, partially precipitable by ammonium sulphate, resistant to mild alkali treatment, heat-labile, soluble in water and isotonic saline, and to give positive tests in Millon-Nasse

and biuret colour reactions.

Nothing can be said as to its origin, or concerning what its physiological significance may be. The daily excretion by a few normal men has usually been found to be equivalent to 40 to 200 mg. of a reference preparation.

The mechanism of action of APU remains obscure, but some observations described above deserve to be commented on. In this respect the most salient result is the difference in behaviour between liver slices and homogenates incubated in the presence of APU. In slices the maximal glucose response to APU was a moderate one and considerable amounts of glycogen remained in the tissue at the end of incubation; while in homogenates the only limitation to the increase in glycogenolysis appeared to be the amount of glycogen present, since a sufficiently high concentration of APU invariably resulted in almost complete disappearance of glycogen. It is therefore necessary in discussing the action of APU on glycogenolysis <u>in vitro</u> to distinguish between these two tissue preparations, and to consider the possibility that the difference in response may be due to more than one substance present in the crude extract. The results presented above appear to justify the statement that APU is a very active glycogenolytic agent when tested on liver suspensions.

When it comes to interpreting its action on liver slices, however, the situation is not quite as well defined. Evidence was presented to show that the increase in glucose secretion by the slice was not always accompanied by a concomitant decrease in glycogen. This observation may be explained by assuming that in the presence of APU,

either the tissue was synthesizing carbohydrate more actively, or else that the slice utilized a smaller proportion of the glycogen broken down. In the event of this last possibility it must be further assumed that, if APU inhibited carbohydrate utilization by the slice, it did so in a manner which did not disturb the overall oxidative processes, as reflected by the oxygen consumption of the tissue. However, no experiments were carried out to investigate what action APU may have on gluconeogenesis. The elucidation of the mechanism of action of APU must await further experimentation, but the results presented above indicate that its mode of action, in homogenates at least, is unlike that of HGF and epinephrine. However, the writer feels that this aspect of the investigation is secondary to the question of whether APU plays a rôle in human physiology.

GENERAL SUMMARY

1. The rôle of the pituitary-adrenal system in the pathogenesis of the diseases of adaptation is reviewed, and the evidence pointing to a hormonal regulation of corticotrophin secretion is presented.

2. Experiments are described with the object of showing that stimulation of the adrenal cortex is possible even in the presence of excessive amounts of cortical secretion, and the implications of these results are discussed.

3. The effect of dietary protein on the responsiveness of the adrenal cortex is discussed.

4. Experiments are described indicating that:

(a) a 30%-protein diet stimulates adrenal regeneration, following unilateral adrenalectomy, to a greater degree than a 15%-protein diet;

(b) rats pretreated with a 30%-protein diet give a greater adrenal ascorbic acid response to acute stress, than animals receiving a 15%-protein regime;

(c) the protein content of the diets studied does not affect the adrenal response of the hypophysectomized rat to exogenous ACTH.

5. A hypothetical explanation of these results is advanced and discussed.

6. The evidence pointing to the existance of a pancreatic hyperglycemic glycogenolytic factor is collected and discussed.

7. Previous investigations in which it is claimed that urine contains a hyperglycemic factor are reviewed.

8. Experiments are presented showing a procedure to concentrate and partially purify HGF from a waste product of the preparation of insulin. An assay procedure is described, and in vitro manometric studies are presented.

9. Experiments describing attempts to show the presence of HGF in urine are reported.

10. Experimental evidence is presented aiming to show that a new hyperglycemic glycogenolytic substance is present in urine. This material has the following properties: non-dialysable, heat-labile, resistant to mild alkali treatment, partially precipitable by ammonium sulphate, soluble in water and isotonic saline, free of uric acid, precipitable from acidified urine by addition of two volumes of ethanol, and gives positive tests in Millon-Nasse and biuret colour reactions.

11. The activity of this material does not appear to be exerted by contaminants such as: HGF, epinephrine, uric acid, calcium ion or growth hormone.

12. A method of assay is described and a procedure to determine the daily urinary output is presented. Figures are given showing the 24-hour excretion of this substance by normal men to be generally between 40 and 200 units. A unit of activity is tentatively defined as the activity found in 1 mg. of a standard reference substance.

13. The possible mechanisms of action of this material are discussed.

SUMMARY OF ORIGINAL WORK PRESENTED

1. Experiments are presented with the aim of showing that the pituitary-adrenal system responds to stress even in the presence of high concentrations of cortical hormone. It was concluded from these results that the concentration of circulating cortical hormone cannot be the only mechanism responsible for the regulation of adrenocortical activity. These observations have been reported (213).

2. Experiments are reported showing that relatively small deviations from the normal diet of the rat influence the responsiveness of the pituitary-adrenal system. A 30%-protein diet was found to be more effective than a 15%-protein regime in:

(a) stimulating adrenal regeneration following unilateral adrenalectomy,

(b) eliciting adrenal response following acute stress.

The protein content of the diet, however, did not affect the adrenal response of hypophysectomized rats to exogenous ACTH, indicating that dietary protein does not act directly on the adrenal cortex. These experiments have been published (214).

3. A method of concentrating and partially purifying HGF from a waste product of insulin preparation is presented, together with results of *in vitro* manometric studies carried out on one of the fractions obtained.

4. Experiments are reported showing the presence of a hyperglycemic glycogenolytic substance in urine. Evidence is presented to show that this substance is not one of the known, naturally-occurring

glycogenolytic agents. This material has been shown to have the following properties:

(a) non-dialyzable

(b) heat labile

(c) resistant to mild alkali treatment

(d) precipitable from acidified urine by ethanol

(e) partially precipitable by ammonium sulphate

(f) soluble in water and isotonic saline

(g) uric acid-free

(h) gives positive Millon-Nasse test

(i) gives positive biuret test.

An assay procedure is presented and the daily excretion by normal men reported.

B I B L I O G R A P H Y.

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