Children, aged 6 months to 4 years, and suffering from protein-calorie undernutrition, who were admitted into the inpatients department of the Institute of Child Health, Calcutta, were undertaken for these studies. A detailed physical examination including dietetic histories were undertaken on each patient, according to the appended proforma. The subjects were then classified into kwashiorkor, marasmus and nutritional edema, according to the appended criteria and according to the classification suggested by Mukherjee (1967).

The general plan of investigations was as follows; On the day of admission the last feed was given to the patient at 8 p.m. At 9 a.m. on the next morning fasting venous and arterial blood were withdrawn from the femoral vein and femoral arteries. Liver biopsy was undertaken at 9:30 a.m. by the transthoracic route with a vim Silverman needle (Silverman, 1954) at the 8th intercostal space on the anterior axillary line without any local anaesthetic. The biopsy was easily carried out in kwashiorkor where 18-40 mg of liver tissue could readily be obtained. In marasmus and nutritional edema, however, the biopsy was difficult and only 8-16 mg of tissue could be obtained in one attempt. There were frequent failures in marasmus and nutritional edema but no failure in kwashiorkor. No second attempt was tried if the first was a failure. There were no casualties which could be directly attributed to needle biopsy of the liver. As a general routine the children received
crystalline penicillin 250,000 units twice daily for 4 days.

After the liver biopsy on the second day after admission muscle biopsy was carried out on the third day. Oral glucose tolerance test was done on the same day as the liver biopsy and intravenous glucose tolerance and epinephrine tolerance were done on the next consecutive days. Children were gradually put on a diet containing 150-200 calories/day and 4-5 gm of protein/day. The edematous patients usually lost their edema by and 7 to 10 days by 3 months they gained around 30% of their edema free body weight. The series of investigations were then repeated on all the children before discharge.

All solvents were either analytical or C.P. grade; and they were redistilled and kept in proper bottles. The trichloracetic acid used was distilled in vacuo. Rabbit glycogen was purchased from Sardar Ballavbhai Patel Chest Institute, Delhi, the different substrates and enzymes were purchased from Sigma Chemical Company, St. Louis, Missouri, U.S.A., the chemicals were all of analytical quality supplied by British Drug Houses, India or England and by Sarabhai Merck, Baroda. Some of the chemicals needed recrystallization or redistillation.

The following methods were used in the various materials of study.
Glycogen content:

Principles - The method consists in the digestion of the tissue in hot conc. KOH, precipitation of the glycogen with ethanol and the determination of glucose by anthrone.

Seifter et al (1950) showed that in tissues such as liver where the glycogen content is high (approximately from 1.5 to 9%) the glycogen can be determined in the presence of proteins. The method is relatively easy; since the necessity for glycogen precipitation and hydrolysis is eliminated. The glycogen is directly determined colorimetrically with anthrone reagent.

Procedure - Just after removal of the liver a portion of the tissues (about 2-7 mg) was cut, weighed and put into a small centrifuge tube containing 0.12 ml of 30% KOH and put on a boiling water bath for 20 minutes. When the tissue was dissolved the tube was taken out and cooled. The glycogen was precipitated by the addition of 1.2 vols. of 95% ethanol. The tube and contents are heated again until the mixture just begins to boil, then it was kept in a beaker containing ice for at least 2 hours. Now it was centrifuged, the mother liquor was decanted and the test tube was allowed to drain. The precipitated glycogen was washed by 60% ethanol twice more.
The precipitate was dissolved in water (about 1 mg per ml) and 2 ml aliquot was taken. Into a second tube was introduced 2 ml of the glucose standard containing 100 µg of glucose. To a third tube was added 2 ml of water, which served as the blank. The tubes were submerged in ice-cold water, 4 ml of the anthrone reagent (2% anthrone in 95% H₂SO₄) was added to each tube and the reactants were mixed by swirling the tubes. The cold tubes were covered with glass marbles and boiled for 10 minutes in a boiling water bath. They were then immediately cooled in a bath containing cold water and read in the Spectrophotometer at 620 nm. The amount of glycogen in the aliquot used is calculated from the following equation.

\[
\frac{100 \times U}{1.11 \times S}
\]

where \( U \) = the optical density of unknown solution
\( S \) = " " " " the (100)g glucose standard

1.11 = the factor determined by Mofris for the conversion of glucose to glycogen, in this equation.

**Glucose-6-Phosphatase**

**Principle** - The method (Her's 1964) is based on the incubation of the specific substrate with the enzyme and determination of the liberated orthophosphate.
Reagents - Glucose-6-Phosphate stock solution (0.1M) 260 mg of the barium salt was suspended in 2 ml of distilled water, and dissolved in the minimum amount of 1 N HCl. 72 mg (0.5mM) of anhydrous sodium sulphate was added. The precipitated barium sulphate was removed by centrifugation, and the supernatant solution was tested for complete precipitation with a very small amount of sodium sulphate. The pH of the supernatant solution was brought to 6.5 with NaOH, and the volume was made up to 5 ml.

Maleic buffer - 116 mg of maleic acid was dissolved in water, and brought to pH 6.5 with NaOH and made to 10 ml.

Procedure - Within 2-3 min. after removal, the tissue was homogenized in a Potter Elvejem - glass homogenizer with 0.05 tris buffer in 0.154 M NaCl (30 mg per ml) in the cold. It was then centrifuged for 1 minute only in 5000 g. Then 0.1 ml of substrate solution and 0.3 ml of maleic buffer was added to a centrifuge tube and brought to 37°C in a water bath. 0.1 ml of homogenate (containing 2 to 5 mg tissue equivalent) was added and mixed by swirling, then incubated for 15 minutes. After incubation one ml of 10% TCA was added to stop the reaction and the whole thing was kept in an ice-bath for some time. Then 1 ml of water was added to make the volume 2.5 ml and was centrifuged. 2 ml aliquots
of the supernatant solution was taken for determination of inorganic phosphate. A small amount of the phosphatase which is formed under these conditions is produced by the action of unspecific phosphatases. This amount can be estimated by running a blank in which glucose-6-phosphate has been specifically destroyed by a short incubation at pH 5.0. 0.1 ml of 1% liver extract was mixed with 0.01 ml of 0.1 M acetate buffer, pH 5, and incubated at 37°C; 5 min later, 0.1 ml of 0.1 M glucose-6-phosphate, pH 6.5, was added and the reaction was run as already described. The amount of acetate buffer is sufficient to ensure complete destruction of the specific glucose-6-phosphatase but does not change the pH during the assay period. The activity was expressed in micromoles of phosphate formed per minute per gram of fresh liver.

PHOSPHORYLASE

Phosphorylase is measured by the liberation of inorganic phosphate from glucose-1-phosphate in the presence of glycogen (Cori et al., 1943).

In the method described by Hers (1964) the substrate contains 0.1M glucose-1-phosphate, 2% glycogen, 0.003 M adenosine monophosphate and 0.2M sodium fluoride, pH 6.1; 0.05 ml of the substrate and 0.05 ml of a 1% tissue extract were mixed in the bottom of centrifuge tube and incubated at 37°C for 0 (trichloroacetic acid added before the extract), 10 and 20 minutes. The reaction was stopped by the addition of 0.5 ml of 1M trichloroacetic acid. It was
cooled (ice-chilled) then 0.5 ml of water was added and centrifuged. The supernatant was taken and mixed with another 1 ml of water and 0.5 ml aliquot was taken for the determination of inorganic phosphate.

1 ml aliquot was taken to which was added 1 ml ammonium molybdate (0.2% w/v) and 1 ml of ascorbic acid (10% w/v) and made up to 5 ml with water. The mixture was incubated at 37°C for 1 hour, and the optical density was measured at 650 μm. A tube containing only water and another containing 4 μg inorganic phosphate (as KH₂PO₄) were treated in the same way.

Glycogen Synthetase -

Total UDP-glucose glycogen transferase activity was determined (Baudouin 1967) by measuring the production of UDP in the presence of UDP-glucose and glycogen.

The uronic diphosphate (UDP) formed during the incorporation of glucose of UDP-G into glycogen is measured by using pyruvate kinase (PK) and phosphoenolpyruvic acid (PEP). The UDP is converted with PEP in the auxiliary reaction to UTP and pyruvate, the latter being determined by means of the decrease in optical density on oxidation of DPNH to DPN by lactic dehydrogenase. In the complete system, for each mole of UDPG that was utilized 1 mole UDP was formed and one mole glucose was added to glycogen.
An aliquot of the homogenized sample (of both liver and muscle) was incubated for 30 min at 30°C, in a total volume of 0.8 ml containing 1.4 μM UDP-glucose, 0.4 mg of glycogen (KOH extracted), 1 μM of glucose-6-phosphate, 10 μM potassium glycylglycine buffer (pH 7.8), 1.6 μM mercaptoethanol, and 0.4 μM EDTA. After incubation, the mixture was heated in a boiling water bath for 1 min. Controls were similarly treated but without incubation.

After centrifugation at 20,000 x g for 15 min, UDP was determined in an aliquot of the supernatant by coupling the dephosphorylation of phosphoenolpyruvate catalyzed by pyruvic kinase, with the oxidation of NADH, and catalyzed by lactic dehydrogenase. The reaction mixtures, in a total volume of 3.12 ml, contained in addition to the sample, 1.2 μM phosphoenolpyruvate, 40 μM potassium glycylglycine buffer (pH 7.4), 15 μM MgSO₄, 45 μM KCl, 0.6 unit of lactic dehydro-dehydrogenase, and 0.1 μM NADH. After the optical density at 340 μm had reached a constant value, 0.8 unit of pyruvate kinase was added and the decrease in optical density was determined after a constant value had been reached (6 to 10 min). UDP-glucose glycogen transferase activity was expressed as micromoles of UDP produced in one minute.

Protein Measurement with the Folin Reagent (J. Biol. Chem. 193, 265, 1951):
Principle:

The principle of the method is that a Copper tartrate complex is allowed to react with the protein in alkaline solution. The protein-copper complex can reduce phosphomolybdate to form a blue substrate with a broad absorption peak around 750 nm.

Reagents:

A) 2% Na₂CO₃ in 0.1 N NaOH

B) 0.5% CuSO₄₅H₂O in 1% Sodium or K tartarate

C) Alkaline copper solution - Mix 50 ml reagent A with 1 ml reagent B. Discard after 1 day.

D) Diluted Folin Reagent - Titrate Folin-Cocalten Phenol reagent with NaOH to a phenapthalein end point.

On the basis of this titration dilute the Folin Reagent to make it 1 N in acid.

Working standard was prepared from bovine albumin.

Procedure: To a sample of 5 to 100 y of protein in 0.2 ml or less in a 3 to 10 ml test tube, 1 ml Reagent C was added. It was mixed well and allowed to stand at room temp. for 10 minute. 0.1 ml of reagent E was added very rapidly and mixed within a second or two. After 30 minute or longer, the sample was read in a spectrophotometer.
BLOOD GLUCOSE (Nelson-Somogyi, 1944, 45)

The methods for the determination of blood glucose are based upon the ability of glucose in hot alkaline solution to reduce certain metallic ions, of which the cupric and ferricyanide are most commonly used. The extent of reduction is then measured by photometric, titrimetric or gasometric methods.

Blood is deproteinized by a zinc sulphate-barium hydroxide procedure which gives a filtrate containing practically no reducing substances other than glucose. The zinc hydroxide filtrate was heated with an alkaline copper reagent and then treated with an arsenomolybdate color reagent. The color developed is compared with that obtained from a known amount of glucose.

Reagents -

Barium Hydroxide Solution. 90 g of \( \text{Ba(OH)}_2 \cdot 8\text{H}_2\text{O} \) was dissolved in distilled water and diluted to 2000 ml in a graduated cylinder. It was filtered, if cloudy, and stored in well-stoppered containers filled to capacity.

Zinc Sulphate Solution. 100 g of \( \text{ZnSO}_4 \cdot 7\text{H}_2\text{O} \) was dissolved in distilled water, diluted to 2000 ml in a graduated cylinder, and mixed. It is stable for indefinite period.
Alkaline Copper Reagent: Solution A. 50 g of anhydrous sodium carbonate, 50 g of Rochelle salt and 40 g of sodium carbonate, was dissolved in about 1600 ml of distilled water, and diluted to 2 liters. The solution was mixed and filtered, if not clear. It was stored at room temperature.

Solution B. 150 g CuSO₄ · 5H₂O was dissolved in distilled water and diluted to 1 liter. 0.5 ml of concentrated sulphuric acid was mixed in it.

Alkaline Copper Reagent. On the day it is to be used, 4 ml of solution B was placed in a 100 ml graduated cylinder, and the volume was made up to 100 with solution A.

Arsenomolybdate Color Reagent: 100 g of ammonium molybdate was dissolved in 1800 ml of distilled water. 84 ml of conc. sulphuric acid was added with stirring. 12 g of disodium orthoarsenate (Na₂H₂AsO₄ · 7H₂O) was dissolved in 100 ml of distilled water and it was added to the acidified molybdate solution with stirring. The mixture was placed in an incubator at 37°C for 1 or 2 days. It was stored in a glass-stoppered brown glass bottle. The solution is stable indefinitely.

Standard Glucose Solution: A stock standard was prepared by dissolving exactly 1.0 g of highest purity anhydrous glucose in about 10 to 15 ml of 0.2 per cent benzoic acid solution, transferring quantitatively to
a 100 ml volumetric flask, diluting to the mark with the benzoic acid solution and mixed. The solution is stable indefinitely, and contains 10 mg of glucose per ml. For working conditions the stock solution is diluted according to the requirement. For example for standard 0.025 mg glucose per 0.5 ml of stock standard was diluted to 200 ml with 0.2 per cent benzoic acid solution and mixed.

Procedure: - 0.4 ml of blood was taken in a tube and 3.8 ml of barium hydroxide solution was added and mixed. Then 3.8 ml of Zinc sulphate was added and mixed. It was then filtered and the filtrate was collected.

0.5 ml of filtrate was taken and 1 ml of alkaline copper reagent was added, and it was covered by marble and placed upright in a boiling water bath for 20 minutes. The tubes were cooled and 1 ml arsenomolybdate reagent was added and shaken till the effervescence stops. Now it was made up to 10 ml with water and mixed throughly. A blank and a standard was prepared by taking 0.5 ml water and 0.5 ml glucose standard solution (containing 0.025 mg glucose) respectively in place of 0.5 ml filtrate and the rest of the procedure is same. The reading was taken at 540 mu. and calculated by the following way:

\[
\frac{\text{Density of Unknown} \times \text{mg glucose in standard}}{\text{Density of Standard} \times 100} = \frac{X}{0.025} = \text{mg glucose per 100 ml blood.}
\]
Oral glucose tolerance test - The reaction to a measured amount of loading of glucose by mouth is ascertained by sugar determination in blood samples collected at regular intervals after ingestion of the dose. A normal, high and prolonged, or low rise of the blood sugar would indicate, respectively, normal, decreased or increased tolerance.

Procedure: The glucose loading dose is given in the morning, after 12 hours fast. The glucose dose commonly used is 1.75 gm per kilogram body weight. The calculated amount of glucose is given in 20 per cent aqueous solution. Capillary blood was taken before 1/3, 1/2, 2 and 3 hours after ingestion of the test dose.

Blood sugar was determined in the blood specimens by the micro-method of Nelson and Somogyi (1944). Proteins were removed by precipitation with a Zinc sulphate barium hydroxide mixture. An alkaline copper tartarate reagent was used as substrate for reduction, and Nelson's arsenomolybdate reagent was used for color production. Color intensity was measured spectrophotometrically. The method has been widely used in children as the most reliable procedure of measuring true blood sugar.

Procedure: 0.1 ml capillary blood was washed from a pipet into 3.5 ml of water contained in a small centrifuge tube. After addition of 0.2 ml each of barium hydroxide and zinc sulphate reagent, the contents were mixed, and after a few minutes centrifuged. Then 1 ml of
the supernatant (± 0.025 ml of blood) was pipetted into a test tube graduated at 10 ml. To this was added 1 ml of water and 2 ml of alkaline copper reagent, readied the same day as described above. In the same way 1 ml of the glucose standard (containing 0.05 mg glucose) and 1 ml of water were set up in two similar tubes and treated with the precipitating and copper reagents. All the samples were heated in boiling water bath for 10 minutes. When cooled, 1 ml of arsenomolybdate reagent and water up to the 10 ml mark was added to each. The optical densities were read in a spectrophotometer at 500 μm.

Calculation: Under the above experimental conditions with the standard containing 0.05 mg glucose

\[
\text{mg glucose/100 ml blood} = \frac{\text{Reading of Unknown}}{\text{Reading of standard}} \times 200
\]

Intravenous glucose tolerance test

When the glucose load is administered intravenously, the tolerance curve is not conditioned by the rate of intestinal absorption, as in the oral tests. This has been generally recognized and appreciated. To judge from recent comments, however, many insist that the intravenous test alone is a useful diagnostic tool. When the results of oral and intravenous tests in the same patient differ, the positive findings are by no means
always those obtained with the intravenous method (Pachman, 1940; Talbot et al, 1952; Wright et al, 1957).

Procedure: The child was prepared as for the oral test. As recommended by Crawford (1938), the standard dose for infants and children is 0.5 gm glucose per kilogram body weight, injected as a 20 per cent solution in 0.9 per cent saline, at a rate of 45 seconds for each 20 ml. Samples of capillary blood are taken just before and 5, 30, 60, 90, 120, and 180 minutes after injection. Blood sugar determination was carried out by the method of Nelson-Somogyi (Nelson, 1944; Somogyi, 1952).

Adrenaline Tolerance Test:

Parenteral administration of epinephrine results in hyperglycemia by causing a more rapid breakdown of liver glycogen and reducing the tissue utilization of sugar. The glycogenolytic action of epinephrine has been utilized for testing the availability of liver glycogen for metabolic oxidation. A hyperglycemic response to epinephrine proves that the liver is able to mobilize sugar from its glycogen stores, while failure to produce such a response reveals a lack of available glycogen.

Procedure:– The test is performed after an overnight fast. The commonly used dose is 0.3 to 0.5 mg epinephrine hydrochloride, contained in 0.3 to 0.5 (5 to 10 minutes) of a 1:1000 epinephrine solution. The dose may also
be calculated as 0.03 cc (1/2 minim) of the 1:1000 solution per kilogram body weight or 0.3 cc (5 minim) per square meter of body surface. Blood samples were collected before and every 30 minutes for 24 hours after subcutaneous administration of the test dose. The blood was analyzed for sugar.

**ESTIMATION OF HYDROXYCORTICOIDS IN THE PLASMA**

**Principle:** Cortisol is extracted from plasma or urine with methylene chloride and estimated by its fluorescence. Only 1 to 2 ml of plasma is required for each estimation and the fluorescence is read in a standard direct reading fluorimeter. A simple fluorimetric method of Mattingly (1961) was followed for estimation of plasma cortisol content. Most of the usual methods were based on the Porter-Silber reaction for 17-hydroxycorticoids of Nelson and Samuel (1952) and Silber and Porter (1954) or on the measurement of the fluorescence of the individual steroids after their separation by chromatography (Sweat, 1954; Lewis, 1957; Bondy et al, 1957; Braunsberg and James, 1960). All these methods required at least 5 ml of plasma for a single estimation which is difficult to draw from a child suffering from malnutrition.

**Reagents:**

A. Methylene Chloride (anhydrous);

It was prepared by mixing tenth volume of concentrated
sulphuric acid and allowing it to stand for 2-4 weeks with occasional shaking; the treatment was repeated until the acid remained colorless. The methylene chloride was washed with 1/10 volume of 1 M sodium hydroxide and then twice with distilled water. It was dried over anhydrous sodium sulfate and distilled. The fraction distilling between 39 and 40° C was collected.

B. Sodium Hydroxide: 0.10 M aqueous.

C. Fluorescence Activation Reagent:

Three volumes of ethyl alcohol were mixed with 7 volumes of conc. H₂SO₄ in a flask which was cooled under a cold water tap. The solution should remain colorless. If a brown color develops, the ethyl alcohol should be purified (Petersen et al, 1955). This reagent is stable at room temperature.

D. Cortisol Standard:

50 mg. cortisol was dissolved in 50 ml of purified ethyl alcohol. 1 ml of this solution diluted to 100 ml with distilled water (10 ug/ml). This solution remains stable for months at 4°C. Standard solution containing 1 ug/ml only remain stable for about a month and prepared when required by further dilution of the 10 ug/ml standard.
Procedure:

1 ml plasma was taken in a separating funnel (50 ml) and 6 ml methylene chloride was added to it. The separating funnel was gently shaken for 20 minutes in a slow mechanical shaker, to avoid the formation of emulsion. The aqueous phase was removed and about 7.5 ml methylene chloride extract was collected in a tube. This extract was poured again into a separating funnel and was washed with 0.1 M NaOH. The alkali wash was repeated to remove the phenolic compounds. Then the organic phase was washed with 2.5 ml water. From the washed extract, 5 ml methylene chloride was taken in a tube, and 2.5 ml fluorescence activation reagent was added in it. It was shaken vigorously for 20 seconds. After 3 minutes, 2 ml of the acid extract was transferred to a quartz cuvette. After 14 minutes (at room temperature) from the time of adding the fluorescence reagent, the fluorescence was then read at 520-540 nm using the activation wave length of 475 nm.

A reagent blank (1 ml distilled water) and a cortisol standard (2 ug) were carried through this procedure with each plasma sample.
ESTIMATION OF INSULIN CONTENT OF THE PLASMA BY
RADIO IMMUNOASSAY

The method depends on the principle first utilized
by Yalow and Berson (1960), that in a reaction with a fixed
quantity of antibody, unlabelled antigen will compete
with labelled antigen for the limited number of binding
sites available. Thus, when serum containing unknown
quantity of insulin is incubated with anti-insulin
antibody in the presence of a fixed quantity of labelled
insulin, the amount of bound labelled insulin will
decrease in proportion to the amount of unlabelled
insulin present in the sample. The extent of binding
can be compared with reference to the addition of known
quantity of insulin used with the same technique and
thus the quantity of insulin in the plasma can be easily
determined. With greater and greater quantity of unlabelled
insulin less and less counts will be observed with the
fixed amount of radioactive insulin present. Since
insulin-antibody complexes are soluble, it has been
necessary to devise some method of separating free
insulin from antibody bound insulin. The separation
was achieved by chromatoelectrophoresis in the method
of Yalow and Berson (1960) and by functional precipita-
tion with salts in the method of Grodsky and Forsham
(1960). In the method introduced by Hales and Randle
(1963), insulin antibody complex is precipitated by the
use of anti y globulin serum. Furthermore, it was found that the insulin antibody which was precipitated with anti-globulin serum was still capable of reacting with insulin. Hence, procedure of Hales and Randle (1963) was followed with some modification, for the assay of insulin content of the plasma by radioimmuno-assay.

The following reagents were used for the assay. They were obtained in the form of a kit supplied by the Radio Chemical Centre, Amersham, England.

1. Iodinated insulin - I\(^{125}\)
Solution in 0.04 M phosphate buffer, pH 7.4, containing 0.6 mM thiomersal and 0.1% bovine plasma albumin. Iodinated insulin was prepared from specially purified crystalline OX insulin (Bourroughs Wellcome & Co., Potency 24.3 international units/mg) by iodination with iodine monochloride, unbound iodine was removed by gel filtration. Each vial contained 0.1 \(\mu g\) of iodinated insulin dissolved in 1 ml of buffer A. The working solution was prepared by adding 0.1 ml of the reagent, with the aid of plastipak disposable syringe, to 3.9 ml of buffer A. 0.1 ml of the diluted mixture contained 250 \(\mu g\) of iodinated insulin.
2. **Insulin Binding Reagent**

Antisera were prepared to crystalline pig insulin (in guineapigs) and to guineapig serum proteins (in rabbits). After adjusting the titre of the anti-insulin serum it was diluted in buffer containing ethylene diamine tetra-acetic acid (EDTA) and mixed with a predetermined amount of the anti-guineapig serum. The mixture was allowed to react at 4°C for 18 hours and then freeze-dried. When reconstituted to a volume of 3 ml (Vide Procedure) each vial contained guineapig serum diluted to 1 in 16,000 and 0.03 M EDTA in buffer A. This reagent used in the present method would bind approximately 40% of the standard dose of 250 µg of iodinated insulin.

It was stored at 2 to 4°C in the dry state. After reconstitution, it was again stored at -20°C for three days which was the maximum time permitted for estimation of the activity.

The stock solution was stored at -20°C in the deep freeze.

**Deionised Water**

All solutions were prepared with deionised water, obtained by triple distillation in all glass vessels.
Buffer A.

Phosphate buffer (40 mM, pH 7.4) containing thiamersal (0.6 mM) and bovine plasma albumin (0.1%) for dilution of antisera and iodinated insulin. The solution was stored at 2° to 4° C.

Buffer B.

Isotonic buffer for dilution of standard insulin or plasma samples.

NaCl, 9.0 g.
Buffer A to 1 liter.

Standard Insulin Solution

The following procedure was applied for the preparation of standard insulin solutions from the pure solid.

(a) First Stock Solution

Condensation of water vapor on the solid protein was avoided by allowing the insulin in its container to warm up to room temperature before opening. About 2 mg insulin was weighed accurately in a small tube and 1 ml of 0.03 M HCl added to it. When solution was complete the whole thing was transferred into a 10 ml volumetric flask with washing and the volume was made up with deionised water. Then 0.1 ml volumes were dispensed in small capped test tubes with the help of a micropipette.
This solution was stored in the deep-freeze. Repeated freezing and thawing of this solution was not permitted.

(b) Second Stock Solution

One of the test tubes of the first stock solution was thawed, 2 ml of buffer B was added, and the solution was transferred with the aid of a Pasteur pipette to a 10 ml volumetric flask. The test tube was rinsed twice more with buffer, the rinsings were added to the flask and make up to 10 ml with the buffer B. This second stock solution could be stored deep-frozen for some weeks without demonstrable deterioration.

Working Standards

An initial working standard was prepared by diluting 0.5 ml of the second stock solution to 50 ml with buffer B; this solution had the concentration of 20 μg/ml. From this initial standard, different concentration of standards were prepared.

Cleaning of Glassware

All glass apparatuses were cleaned by allowing it to stand overnight in chromic-sulphuric acid solution, followed by very thorough rinsing rinsing with tap water. After cleaning, the glasswares were soaked overnight in distilled water and a third time in deionised water.
Immediately before use, the pipettes and syringes were rinsed with buffer A or buffer B, as the case may be.

Procedure (Method of Hale and Randle).

Insulin, Insulin$^{125}$ and anti-insulin serum when reacted together to form a soluble complex. This complex can be isolated by the use of a second antibody, which is an antiguineapig gammaglobulin prepared in rabbits. The precipitates thus obtained can be isolated by filtration or centrifugation and the radioactivity of the precipitate can be measured.

1. Reconstruction of insulin binding reagent.

To one bottle of insulin binding reagent 8 ml of deionised water was added and mixed carefully, avoiding foaming. 0.1 ml volumes were dispensed into precipitin tubes with the aid of micropipette. Each bottle of the reagent was thus sufficient for up to 80 tubes.

Sufficient tubes were set up for:

(a) "Zero", to which no unlabelled insulin was added;
(b) the standard insulin solutions; and
(c) the unknowns.

Each sample was assayed in triplicate, one tube containing only buffer A (without insulin binding reagent) was included in each run as controls of washing procedure.
Addition of unlabelled insulin.

0.1 ml of insulin containing solution (standard or plasma) or 0.1 ml of buffer B for the zeros, were added with the aid of a micropipette. The contents of the tubes were mixed by means of a finely pointed glass rod, then the tubes were placed in a refrigerator at 2 to 4°C for six hours.

Addition of labelled insulin.

After six hours incubation, the contents of each tube were mixed carefully and then 0.1 ml of the working solution of iodinated insulin (250 μg) was added in each tube from a micropipette. After mixing the tubes were returned to the refrigerator for 18 hours.

A sample of the working solution of iodinated insulin was taken for determination of the total radioactivity added to each tube.

The precipitates formed in the tubes after incubation were centrifuged at 2°C and the supernatant reaction mixtures were decanted off. The precipitate in each tube was washed thrice with buffer C and each time centrifuged in cold. Then the radioactivity of
these unfiltered sample were measured in scintillation counter.

Since we had no millipore filter discs and millipore microanalysis filter holder, the above modifications were applied in this method by us.