Part II

Material and method of work.

PART II

Materials and Methods of work

(1) Selection of subjects and the General outline of work:

Healthy adult students, mostly medical, between the ages of 18 and 24 with no history of serious disease in the past or digestive troubles, were given a clinical overhaul and selected as subjects after their fractional gastric analysis with alcohol meal (advantages of this meal will be discussed later) and evacuation time with barium meal were found to be normal. The fractional examination was done with alcohol meal as described by Bloomfield and Koofer (1926) and a week after this, four ounces of barium suspended in water was given and the type of the stomach, its outline and evacuation time, were determined. After selection as subjects, the persons were called once in a week, in the early morning, without food and were made to rest and then given the article of food usually in 100 gms. portion. Levin's tube was then passed into the stomach usually by the subject himself, and was taken out by him at the end of the experiment. In a preliminary work, Levin's tube was found to be more suitable than the usual Ryle's tube for aspirating the contents and for introducing it into the stomach also. The subjects were thoroughly used to the tube before starting the experiment. A few subjects, otherwise normal, but sensitive to the tube had to be rejected. Standardization regarding the environment, mental state and such factors was strictly followed. Any minor but abnormal factor, for example, the subject had a "cold in the head", or otherwise did not feel well or the subject had any cause of worry or anxiety,

was inquired into and if any such factor likely to affect the result was noticed, the experiment was postponed to a future date. Cheerful atmosphere was maintained and the subjects were allowed to move about, read papers and light literature and to communicate with others.

First sample was aspirated 15 minutes after the article was given and subsequently half-hourly till clear material with no evidence of food was obtained. This was taken as the evacuation time by the fractional method. The aspirated material was examined for consistency of food particles, colour, mucus, bile, degree of free and total acidity, total chlorides (in some cases), presence of peptones and in some cases for starch and reducing substances and for any other abnormality like blood etc.

A few samples of some of the articles of food which were given to the subjects, were analysed for their chemical composition with respect to the contents of proteins, fats, moisture, ash and carbohydrates (by difference). This chemical analysis was done with a view to find out if any relation could be obtained between their composition and the gastric secretion as it was thought that the composition of the cooked articles might be differing from that of uncooked articles; while the values of the composition of the uncooked articles are generally available from the tables.

(3) Methods:

Introduction and the choice of the method of Fractional Analysis:-

While it is true that we have certain conceptions regarding the digestion of the various groups of foodstuffs, our knowledge, on this subject has been more or less based on Roentgen ray observations on the study of individuals with gastric fistulae, or other

-wise traumatized, or by means of a method, namely the old form of intubation, which did not permit of satisfactory study, of the entire period of digestion. By recourse to fractional analysis, Hawk, Rehfuss and Bergeim (loc cit) were able to follow the digestion of various foodstuffs throughout the entire period of gastric digestion and they were enabled to study precisely the important points regarding their evolution. The authors, Hawk et al (loc cit) have stated, "This series of studies, performed on a large number of normal persons, all of whom were completely habituated to the use of the tube, represents, to our knowledge, the first complete series of investigations covering the action of all the different varieties of foodstuffs in the normal human stomach."

The fractional method of gastric analysis:

Choice of the stomach tube:-

The method of gastric analysis in the beginning which was in vogue clinically for years entailed the feeding of a standard test meal, the removal of the complete stomach contents at the end of a one-hour period and the analysis of the material so removed. That this method was inaccurate had been repeatedly demonstrated by Rehfuss (1914), Rehfuss, Bergeim and Hawk (1914), Bergeim, Rehfuss and Hawk (1914) and Hamer and Dodd (1913). Furthermore, owing to the bulk of the old form of stomach tube, and the discomfort occasioned by its use, it was impossible to follow the whole cycle of digestion and to estimate, step by step, the exact changes which take place in the stomach after the introduction of definite food mixtures into the organ.

Realizing the inadequacy of the procedure entailed in the above mentioned old method of gastric analysis, a new procedure

was developed by Rehfuss (loc cit). This so called "Fractional Method" entails the analysis of samples of material withdrawn from the stomach (by syringe) at short intervals for a period of two hours or more until emptying of the stomach, after the ingestion of the test meal. By this means the observer is able to follow the entire cycle of gastric digestion and is not limited, as in the old method, to information derived from the analysis of a single sample of stomach contents withdrawn at the end of one hour.

The removal of samples of gastric contents at short intervals, for a period of two hours or more after a test meal has been made possible by the use of a modified stomach tube (Rehfuss, 1914) of small diameter and fitted with a metal tip. The tip is slotted with large perforations, the diameter of each being equivalent to the maximum bore of the tubing. Such a tube can be left in the stomach through the entire cycle of gastric digestion without inconvenience to the subject. A tube much favoured in England is that devised by Ryle (1926). This consists of a small bore rubber tube with a blind end, into which is inserted an oval weight of lead. Holes are punched in the rubber tube just above the weight. Ryle (loc cit) claimed the following advantages over a metal-tipped tube: (1) more easily swallowed and withdrawn; (2) blockage with mucus plugs generally avoided; (3) impossible to detach end; (4) less likelihood of damaging the gastric mucosa; (5) cheaper.

Levin's tube which is differing from the Ryle's tube in the respect of having a larger bore and having no lead weight has also the same advantages as stated above over the Rehfuss tube. In our experience, Levin's tube was found to be more suitable than Ryle's tube because of its larger bore no blockage with the food particles, especially when solid food articles were ingested, was taking place, which used to

occur more often with the Ryle's tube.

Introduction of the Stomach tube:

The tube is swallowed in the natural manner with the aid of gravity. The tube may be passed in one of the three ways, viz.:(1) lubricated; (2) with aid of fluid; (3) after throat is cocainized. When passed by the first method the tip of the tube, after through lubrication with glycerol or liquid petrolatum, is held between the thumb and forefinger and placed on the tongue. Then with the aid of the forefinger the tip is pushed backward until it reaches the root of the tongue and is engaged in the oropharynx. Then the subject is encouraged to breathe deeply through the nose and to swallow persistently while the tube is slowly fed into the mouth. After slight discomfort in the pharynx and its passage past the level of the cricoid cartilage, practically no discomfort is felt. This method is used when it is essential that the pure gastric secretion or residuum be obtained. Ordinarily, however, it is much easier to swallow the tube by the second method. This method consists in placing the tip in the oropharynx and then giving the subject a measured quantity of water or tea to swallow. The movements induced by the swallowing carry the tube rapidly to the stomach with a minimum of discomfort. When an Ewald meal (this will be referred to later on) is given, part of the tea may be reserved for swallowing the tube. This procedure makes it scarcely more ardous than the swallowing of food. Should the subject, however, be extremely neurotic or the unfortunate possessor of marked pharyngeal hypersthesia, cocaine hydrochloride in 2 percent aqueous solution may be applied to the throat rendering the passage of the tube practically insensitive.

Out of these three methods the first method e.g. lubricated was adopted in our studies because it was essential in our procedure to

examine the residuum whenever such estimations were carried out and further more in our series some articles of food given to the subjects were in solid form without any liquid counterpart. And it was desirable to keep the same standard procedure constant throughout the whole series of our experiments, which fact naturally made us use the first method even at the time of giving liquid foods also.

After the introduction of the tube, it was seen that the tip reached the lower pole of the stomach. This was made obvious from the fact that when the tube has entered the stomach, aspiration of the material showed the characteristic gastric contents. Should the tip remain in the oesophagus through transient cardiospasm or other cause, aspiration results in the removal of only a very small specimen having all the characteristics of the pharyngeal and oesophageal secretions. Furthermore, the tube was allowed to remain in the position of reaching the lower pole of the stomach throughout the duration of the test, because by placing the tube in this manner, representative specimens and accurate data were enabled to be obtained.

Removal of Residuum:

If the so-called "empty" stomach is examined in the morning before any food or drink has been taken, it will be found to contain considerable material. This is termed residuum. Before a test meal is introduced into the stomach, this organ should be emptied. If this is done, it can be considered that the samples withdrawn after the test meal is eaten as probably representing the secretory activity of the gastric cells under the influence of the stimulation of the test meal. In our studies this procedure was adopted whenever possible, especially in case of the articles of food which were liquid in form but in case of some of the solid type of articles of food it was not adopted becau

it was feared that it would have caused a little more discomfort to the subjects in view of the fact that the tube would have been required to be introduced twice during a very short interval of time, once for taking out the residuum, after which the tube ought to have been withdrawn and again to be reintroduced after the solid test meal was ingested. In case of liquid type of articles of food, this trouble was avoided because after the withdrawal of the residuum, it was possible to introduce the fluid article through the tube itself by keeping the tube in the stomach. Of course in case of some solid type of articles of food, investigations have been carried in the residuum also, in which case the introduction of the tube had to be done twice as stated above.

Technique of removal of gastric contents:

It is important to make a mention here of some of the precautionary measures which were to be taken while removing the gastric contents after the test meal was given.

(1) The subject was supplied either with a sputum mug or a beaker (or any other convenient vessel) into which he was instructed to expectorate in the intervals while tube was being passed and while it remained in the stomach. Under any circumstances, the subject was not allowed to swallow his saliva, the importance of which can be realised from the fact that swallowing of the saliva will affect the chemical composition of the gastric contents, like the acidity etc. or it will cause dilution of the gastric contents.

(2) The subject was not allowed to drink water (except when water was given along with some full meals, etc.) nor water was used to facilitate removal of gastric contents, because this would introduce an unknown dilution.

(3) After withdrawing of about 10 cc samples of gastric contents by aspiration, at intervals of time after the test meal was given until

the stomach was empty, a few cubic centimeters of air was every time blown down the tube in order that the tube should be empty when the next sample was drawn. Otherwise the first 1-2 cc aspirated the next time may consist of the previous sample.

(4) In order to facilitate the mixing of the stomach contents and the withdrawal of a more representative sample the stomach contents were mixed by physical manipulation just prior to aspiration. The mixing of the stomach contents can be achieved by aspirating them back and forth four times before taking the sample for analysis.

(5) In the removal of the samples from the stomach, it was essential that very little traction be employed. When no more material could be aspirated in any of the positions like, on the back, or on the stomach or on the left side or on the right side, was taken to be completely emptying of the stomach.

Ehrmann Alcohol Meal as the Test Meal:

As it has already been stated previously, before selecting the subjects for this study they were subjected to the fractional gastric analysis examination with alcohol meal. A few remarks regarding the choice of the alcohol meal as the test meal will not be out of place here.

The procedure of fractional gastric analysis constitutes a means of studying the manner in which the stomach responds to stimulation. The stimulation may be artificial, as represented by the injection of histamine or the administration of alcohol, or it may be natural in the form of various foodstuffs introduced into the stomach. Each form of stimulation possesses advantages under certain circumstances, the injection of histamine being of particular value in obtaining a maximum secretory response and in establishing a diagnosis of true achlorhydria, the alcohol meal being of value in cases in which the quantitative

determination of the volume of gastric secretion is desirable. The ordinary foodstuffs meals possess the advantage of yielding information concerning gastric digestion and the motor activity of the stomach.

A mention may be made of some of the important test meals which are generally used in practice. They are (1) Ewald test meal consisting of wheat bread or toast weighing approximately 35 gm. and eight ounces of water or weak tea without sugar or cream; (2) Boas meal consisting of one table spoonfull of oatmeal boiled in 300 cc. of water until the volume is reduced to 400 cc.; (3)Riegel meal consisting of about 200 cc. of beef broth, 150-200 gm. of boiled beef steak and about 100 gm. of mashed potatoes.

The Ehrmann Alcohol Meal which was modified and recommended by Bloomfield and Koefer (loc cit), consists of 50 cc. of 7 percent alcohol. This meal possesses the following advantages over the other meals mentioned above:-

(1) It has the readiness of administration .
(2) It provides the ease of the withdrawal of samples .
(3) It furnishes the ease of examination of specimen .
(4) It makes possible a more exact quantitative determination of the volume of gastric juice.

(A) Examination of the samples of the stomach contents:

The samples of the stomach contents were examined for the following constituents:-

(a) Free acidity content
(b) Total acidity content
(c) Total chloride content
(d) Presence of lactic acid
(e) Presence of occult blood

(f) Presence of mucus (consistency)

(g) Presence of bile

(h) Presence of starch

(i) Presence of reducing sugar

(j) Presence of peptones

(k) Presence of proteins

(l) Measurement of the total volume.

(a) Determination of free acidity content:

By the use of Töpfer's reagent (Hawk and Bergeim, 1942)

Reagents:-

(1) Töpfer's reagent (0.5 percent solution of dimethylaminoazobenzene in 96 percent alcohol)

(2) 0.1 N. Sodium hydroxide solution

Procedure:

5 cc. of the strained stomach contents were measured by means of an ostwald pipette and were introduced into a low form 60 cc. porcelain evaporating dish. (In case the quantity of the stomach contents withdrawn was not sufficient enough to have the various analytical procedure -s performed, smaller quantity e.g. 2.5 cc. was used instead of 5 cc. quantity). This was diluted with 10 cc. of distilled water. (If the quantity of the stomach contents taken was less than 5 cc., more quantity of distilled water was added so as to make up the volume to 15 cc.). Then 2 drops of Töpfer's reagent were added and the contents were properly mixed by stirring with the help of a stirring rod. After proper mixing the mixture was titrated against 0.1 N. sodium hydroxide solution to an orange yellow colour. The colour sequence is red, orange and yellow. The titration is complete when reddish orange gives place to yellow orange i.e. when there is no longer any red tinge (indicating that free hydrochloric acid is neutralised). The burette

reading was taken and free acidity was calculated in terms of cc of N/10 acid per 100 cc of gastric contents. It is inaccurate to express results as so many cc of N/10 sodium hydroxide per cent though the burette reading gave the values for N/10 sodium hydroxide, because gastric juice does not contain sodium hydroxide. This last notation, however, is, unfortunately, used very often even in some of the text-books or medical literature. After these results are calculated, the curves showing the free acidity in cc of N/10 acid per cent against time in hours can be drawn.

(b) Determination of total acidity content:

By the use of phenolphthalein indicator method (Hawk and Bergeim (loc cit))

Reagents:-

(1) Phenolphthalein indicator (1 percent alcoholic solution of phenolphthalein)

(2) 0.1 N. Sodium hydroxide solution

Procedure:

When the end point in the determination of the free acidity content (method (a) above) was obtained, which was shown by the yellow orange colour and which indicated that the free hydrochloric acid was neutralized, to the same titration mixture now about 2-3 drops of 1 % alcoholic solution of phenolphthalein indicator were added and the titration was continued against 0.1 N. sodium hydroxide solution which was taken in the burette for the method (a), till the faint pink colour was obtained replacing the yellow colour of the titration mixture. This faint pink colour which was obtained should persist for about two minutes. This reading gave us the neutralization point of the total acidity content. The burette reading, from the commencement of the titration for free acidity, was taken giving the number of cc of N/10 sodium hydroxide required for neutralising all the total acidity content which naturally

included the amount of N/10 sodium hydroxide required to neutralize the free hydrochloric acid also. Here again the total acidity was calculated in terms of cc of N/10 acid per 100 cc of gastric contents. Finally the curves showing the total acidity in cc of N/10 acid per cent against time in hours are drawn.

(c) Determination of total chloride content:

By the use of the modified method of Whitehorn-Volhard (Cole, 1949)

Reagents:-

(1) Standard silver nitrate solution with a strength such that 1 cc of it corresponds to 1 mg. Cl.

(2) Standard potassium thiocyanate solution. Because thiocyanates are hygroscopic the standard solution was prepared volumetrically, with a strength such that 1 cc of this potassium thiocyanate solution corresponds to 1 cc of the standard silver nitrate solution.

(3) Concentrated nitric acid (A.R.)

(4) Ferric alum indicator (saturated solution of ferric ammonium sulphate)

Procedure:

Two cc of the strained stomach contents were introduced by means of an ostwald pipette into an evaporating dish. Then an excess of the standard silver nitrate solution in a quantity of 10 cc measured by means of a volumetric i.e. pipette was added, to which mixture 5 cc of concentrated nitric acid were also added. This was stirred well and the whole mixture was allowed to stand for about five minutes, for the silver chloride to aggregate, which had been precipitated in presence of strong nitric acid. After the silver chloride had thus aggregated, the excess of silver nitrate would now be titrated with standard thiocyanate in the presence of iron alum indicator, which would give a red colour of ferric thiocyanate when the last trace of silver has been precipitated by the thiocyanate. Therefore after the lapse of

about 5 minutes allowed for the aggregation of silver chloride, 1 cc of saturated solution of the ferric alum indicator was added to the whole mixture, which was then titrated with the standard thiocynate solu-tion taken in a 5 cc microburette until a pink colour was obtained which was permanent for 15 seconds. Stirring too much vigorously was avoided once the fluid has been thoroughly mixed with the thiocynate added because vigorous stirring increases the surface of silver chloride and accelerates the disappearance of the pink colour. The burette reading was taken from which the amount of the thiocyanate required for the neutralization of the excess of the silver nitrate was known. As the strengths of the thiocyanate and the silver nitrate solution were such that one cc of the one was equivalent to one cc of the other, the amount of silver nitrate used for precipitating the chlorides in the stomach contents was also easily calculated. Finally the total chloride content in terms of mg. of chlorides per 100 cc of the stomach contents was found out; and the curves showing the total chloride content in mg. of chlorides in 100 cc of the stomach contents against the time in hours were drawn.

(d) Presence of lactic acid in the stomach contents:

By the use of Uffelmann's test (Harrison, 1949)

Reagents:-

(1) Uffelmann's reagent (prepared by treating 1 per cent solution of phenol with very dilute ferric chloride till the solution becomes coloured an amethyst-violet).

Procedure:

The gastric filtrate was added drop by drop to about 10 cc of Uffelmann's reagent taken in a test tube till no further colour change took place. If lactic acid was present in the stomach contents a canary-yellow colour due to ferric lactate was obtained instead of the original violet colour of the reagent.

(e) Presence of occult blood:

By the use of Benzidine reaction (Harrison,(loc cit))

Reagents:-

(1) 0.1 N. sodium hydroxide solution

(2) Benzidine powder (pure grade quality)

(3) Glacial acetic acid

(4) Hydrogen peroxide (ten volumes).

Procedure:

About 3 cc of gastric fluid was taken in a test tube taking care to include any red or brown specks which looked like blood and it was boiled. After cooling, it was neutralised by means of 0.1 N. sodium hydroxide, if acid, using litmus paper as external indicator. In another perfectly clean test tube, a large knife-point of benzidine was taken, to which were added 2-3 cc of glacial acetic acid. It was shaken well to obtain a saturated solution. If all benzidine was dissolved completely, a little more was added till after shaking a few specks of undissolved benzidine were present. A few specks of undissolved benzidine do not interfere but if the solution is not saturated, the test is not so sensitive. An equal volume of "ten volumes" hydrogen peroxide was added and shaken. The benzidine plus peroxide solution was watched to see whether a green or blue colour developed owing to the tube not being really clean. Some of the suspension of the gastric fluid was added to the benzidine plus peroxide solution drop by drop, shaking after each addition and noting any colour change. If necessary an equal volume of the suspension of the gastric fluid was added. A green or blue colour, which often developed rather slowly, resulted if blood was present.

The benzidine reaction may not succeed in suspensions containing free hydrochloric acid, and hence was the necessity for neutralising.

If there is any product of red meat in the gastric contents, the test will be positive apart from blood.

The benzidine is really too sensitive when applied to gastric contents. It must be mentioned here, however, that positive results are very often obtained which are probably due to trauma by the stomach tube.

(f) Presence of mucus (consistency):

This is seen, if present, in the gastric contents with naked-eye examination, which is readily recognized by its ropy appearance.

(g) Presence of bile:

Bile may be obvious from the naked-eye appearance, of course, with exception of cases where the colour of some of the articles of food-stuffs themselves might have interfered with the colour of the bile, if present in the stomach contents. The yellowish or greenish colour of the bile if present in the stomach contents is changed to bright green on allowing the contents to stand for some time. The green colour is due to biliverdin, which has been formed from bilirubin by the action of oxygen of the air on the latter.

If Boldyreff's (1914) theory as to the automatic regulation of gastric acidity under normal conditions by the regurgitation of the alkaline material from the intestine, is accepted, then the presence of bile in the gastric juice does not possess the necessary significance it has been accorded. It should be pointed out, however, that occasion-ally the presence of bile is seen in normal gastric contents which is due to the regurgitation.

(h) Presence of starch:

By the use of the iodine reaction (Harrison (loc cit)):

Reagents:-

(1) N/50 iodine solution

Procedure:-

To a few drops of unfiltered gastric contents taken in an evaporating dish N/50 iodine solution was added drop by drop and shaken. A blue colour demonstrated starch, which might be in solution or enclosed in some of the white lumps in suspension.

(i) Presence of reducing sugars:

By the use of Benedict's test(Hawk and Bergeim (loc cit))

Reagents:-

(1) Benedict's qualitative reagent (for sugar testing)

Procedure:-

To 5 cc. of the Benedict's reagent (qualitative) in a test tube, 8 drops of the strained stomach contents were added. The mixture was boiled vigorously for two minutes and the fluid was allowed to cool down spontaneously (cooling was not hastened by immersion in cold water) In the presence of a reducing sugar the entire body of the solution was filled with a colloidal precipitate, which was either red or yellow or green in colour, depending upon the amount of the reducing sugar present.

In the presence of over 0.2 - 0.3 per cent of the reducing sugar like glucose, the precipitate would form quickly. If no reducing sugar is present, the solution would remain perfectly clear. Even very small quantities of the reducing sugar like glucose (0.1 per cent) would yield precipitates of surprising bulk with Benedict's reagent, and the positive reaction for the reducing substance would be filling of the entire body of the solution with a precipitate, so that the solution would become opaque. Since the amount rather than the colour

of the precipitate has been made the basis of this test, this test had the advantage of being applied even for the detection of small quantities of the reducing sugar, like glucose, in the gastric contents, as readily in artificial light as in daylight.

(j) Presence of peptones:

By the use of the Biuret Test (Cole (loc cit))

Reagents:-

(1) Ammonium sulphate

(2) 40 per cent sodium hydroxide solution

(3) 1 per cent copper sulphate solution

Procedure:

A few cc of the gastric contents were boiled in a test tube. Some solid ammonium sulphate was added and boiled again. Enough of the solid was added to saturate the solution. This mixture was filtered and the filtrate was used for the biuret test. The peptones, if present in the gastric contents, will now be obtained in the filtrate above because peptones are very soluble proteins of rather a low molecular weight and they are the only proteins not precipitated by full saturation with ammonium sulphate. In the filtrate which was obtained above the presence of the peptones, if present, was demonstrated by obtaining a pink colour when some, 40 per cent sodium hydroxide and a drop of 1 per cent copper sulphate solution were added. It should be noted that the biuret test will not be successfully obtained, in this case, unless a large amount of strong soda is used. The solution was saturated with ammonium sulphate, which reacted with the soda to form sodium sulphate and free ammonia. Any undecomposed ammonium sulphate would react with copper sulphate to form the complex $(Cu(NH_3)_4).CuSO_4.H_2O$, thus preventing the copper from reacting to give the pink compound with the peptones.

(k) Presence of proteins:

By the use of the metaphosphoric acid test (Cole (loc cit))

Reagents:-

(1) 25 percent solution of metaphosphoric acid

Procedure:

To a few cc of the gastric contents, a drop or two of 25 per cent solution of metaphosphoric acid were added. The presence of proteins was indicated by the appearance of a white precipitate which was produced, when proteins were present in the gastric contents.

((Note on the preparation of metaphosphoric acid solution:- The metaphosphoric acid is prepared by scraping a stick of glacial phosphoric acid from any loose matter on the exterior, weighing out 2.5 gm and dissolving this by crushing it in a morter with 3 cc of distilled water. The solution is stable for about 4 days. It is most efficient when it has been made up for 6 to 24 hours. The freshly prepared solution is hardly acid enough to precipitate the proteins, but as the metaphosphoric acid (HPO_3) changes to orthophosphoric acid (H_3PO_4) it becomes efficient, to lose this property when the solution has completely changed.))

(1) Measurment of volume:

Total volume of the stomach contents withdrawn on empty stomach before the test meal was given, in other words the examination of the residuum with respect to its total volume, was determined by measuring it accurately in terms of cubic centimeters.

(2) Examination of the extracts in water of the articles of food:

The samples of the articles of food in small quantities were extracted in cold distilled water by the usual methods of trituration with the help of a morter and a pestle and it was then filtered till clear filtrate was obtained.

This cold water extract was analysed qualitatively for the follow -ing constituents:-

(m) Presence of lactic acid

(n) Presence of starch

(o) Presence of reducing sugar

(p) Presence of peptones

(q) Presence of proteins.

Procedures:-

(m) Presence of lactic acid:

Procedure:-

The details of the method are the same as in the case of the exami -nation of the gastric contents i.e. the method (d) above, with the exception that the cold water extract is to be substituted for the gastric contents.

(n) Presence of starch:

Procedure:-

The presence of starch in the cold water extract was determined by the use of the same procedure as described in the case of the examina-tion of gastric contents i.e. the method (h) above. Here the cold-water extract before being filtered was taken in the evaporating dish instead of the unfiltered gastric contents.

(o) Presence of reducing sugar:

Procedure:-

Here again the procedure followed for the determination of the presence of the reducing sugar in the filtered cold-water extract of the articles of food was the same as in the case of the examination of the stomach contents, by the use of the Benedict's test, which is described in the method (i) above.

(p) Presence of peptones:

Procedure:-

The filtered cold water extract of the articles of the food-stuffs

was analysed qualitatively for the presence of the peptones, if any, contained in the extract, by making use of the same method as was used for the examination of the stomach contents, the details of the procedure of which have been described in (j) above.

(q) Presence of proteins:

Procedure:-

The proteins, if present, in the filtered cold water extract of the articles of the foodstuffs, were tested for qualitatively by following the same procedure, which was used for the examination of the stomach contents, as described in the method (k) above.

(C) Examination of the samples of the articles of food:

Samples of some of the articles of food were analysed chemically for the quantitative estimation of their contents as follows:-

(r) Moisture content

(s) Ash content

(t) Proteins content

(u) Fat content

(v) Carbohydrate content (by difference).

Procedures:-

Preparation of a sample of the article of food:

From the articles of food like "Khichdi with milk" or "Full Meal" or "Full feast dish", the aliquot samples were first prepared by taking the various constituents in the same proportion as were eaten by the subjects and then by mixing them well in a homogeneous form. Other articles of food which were solid in nature were also first converted into a homogeneous form by properly mixing them in a mortar and a pestle, before they were weighed out for the analysis of their various constituents. These analyses were made in duplicate and the average was taken.

(r) Determination of the moisture content:

Procedure:-

Two gms. of the sample, of the article of the food, which was prepared as above, was weighed out very accurately in a platinum crucible provided with a close-fitting slip-in lid. The sample was then partially dried on steam bath in the loosely-covered crucible, which was later on placed in a dry-air oven at a temperature slightly above that of boiling water. It was dried to a constant weight, taking care at the time of each weighing that the crucible was covered tightly with the lid before taking out from the oven for weighing. From the loss in weight, the moisture content was calculated in terms of gms. per 100 gms of the sample of the article of food.

(s) Determination of Ash content:

Procedure:-

After the moisture content was determined from the loss in weight according to the procedure just described above in the method (r), the same contents were used for the determination of the ash content in the sample. The contents therefore were ignited very cautiously to avoid spattering on a burner. The burner was removed while the fat, if any, contained in the sample of the article of the food, was burning. When the flame caught by the contents had died out, complete ignition was achieved by keeping the crucible with the contents in a muffle furnace at a temperature not exceeding 550^{o}c. The white ash obtained was weighed very accurately and the ash content was calculated in terms of gms. per 100 gms. of the sample of the article of food.

(t) Determination of the protein content:

By the use of the Kjeldahl's method (Methods of Analysis A.O.A.C. 1950)

Reagents:-

(1) Copper sulphate ($CuSO_4.5\ H_2O$)

(2) Sulphuric acid concentrated pure (A.R.)

(3) 40 per cent sodium hydroxide solution

(4) 0.1 N. Sulphuric acid

(5) 0.1 N. Sodium hydroxide

(6) Methyl red indicator.

Procedure:-

Very accurately weighed 0.7 gm. of the prepared sample was taken in a digestion flask (Kjeldahl), to which were added 10-20 cc of concentrated sulphuric acid and about 0.1 gm of copper sulphate. The flask was placed in an inclined position and was heated below the boiling point of the acid until frothing had ceased. The heat was increased until the acid boiled briskly and the digestion was carried out for a time after the mixture was colourless or nearly so or until the oxidation was completed. This operation was performed in a fuming chamber.

The flask was allowed to cool and the contents were very carefully transferred, after diluting with about 100 cc of ammonia-free distilled water, to a distillation flask and all the washings of the digestion flask with totally about 100 cc ammonia-free distilled water were added up to the contents in the distillation flask. A little coarse pumice stone was introduced into the distillation flask, in order to prevent bumping and a little more of a concentrated (40 per cent) solution of sodium hydroxide than was necessary to neutralise the sulphuric acid, was added very carefully by pouring it down on the sides of the flask, so that it did not mix at once with acid solution, in the distillation flask. In order to know whether the quantity of the sodium hydroxide solution added was sufficient enough to remain in excess after neutralization of the sulphuric acid, was determined by having "check" tests made previously to determine the volume of the sodium hydroxide solution necessary to neutralize the volume (say 10 cc) of the concentrated sulphuric acid used. Secondly a small piece of litmus paper was introduced into the distillation flask prior to the adding of the sodium hydroxide solution to the distillation flask, to ensure the addition of an excess of the sodium hydroxide solution. A known volume i.e. 20 cc

of a standard (0.1 N.) sulphuric acid solution to which were added a drop or two of methy red indicator was taken in a receiving flask and the solution was diluted to about 50 cc with ammonia-free distilled water. About 7-8 cc of this diluted solution of the standard acid were transferred to a catch-bulb which was attached by means of a connecting tube to the receiving flask. The distillation flask was then connected to the condenser by means of a safety-tube (Kjeldahl connecting bulb). The condenser was so arranged that the delivery tube passed into the receiving flask. Care was taken to see that the end of the delivery-tube reached beneath the surface of the liquid in the receiving flask. This delivery-tube should be of a large calibre in order to avoid the "sucking back" of the fluid. The contents of the distillation flask were very thoroughly mixed by shaking, whereupon the litmus paper would be turned blue indicating that the contents in the distillation flask had turned into an alkaline reaction. The mixture in the distillation flask was then distilled until all ammonia had passed over into the measured quantity of the standard acid, which was indicated by the fact that the volume of the mixture in the distillation flask had diminished about one-half. The partly neutralized 0.I.N. sulphuric acid solution was then titrated by means of 0.1 N. sodium hydroxide solution and the burette reading was noted. The number of the cubic centimeters of 0.1 N. sodium hydroxide solution used in the titration was subtracted from the number of cubic centimeters of 0.1 N. sulphuric acid taken. The remainder was equivalent to the number of the cubic centimeters of 0.1 N. sulphuric acid, neutralized by the ammonia evolved during distillation from the ammonium sulphate formed by the action of the concentrated sulphuric acid during digestion, as a result of the conversion of the various nitrogenous bodies; i.e. proteins present in the sample of the article of the foodstuff. By knowing that one cc of 0.1 N. sulphuric acid is equivalent to 0.0014 gm of nitrogen, the total nitrogen content in terms of gms.

per 100 gm of the sample of the article of the food was calculated. The nitrogen content thus obtained was multiplied by the factor 5.7 to obtain proteins content in terms of gms. per 100 gm of the sample of the article of food, if the food sample was of a vegetable type of product, while the factor used for multiplying the nitrogen content in the samples of the articles of food which were of an animal origin, was 6.25, to obtain proteins content in terms of gms. per 100 gms of the sample of the foodstuff. In case of milk and milk products, the factor used was 6.37. No doubt, the usual factor employed for the calculation of protein from the nitrogen content is 6.25 and is based on the assumption that proteins contain on the average 16 per cent of nitrogen. The special factor of 6.37 is used to calculate the protein content from the total nitrogen, in case of the milk and the milk products, since the principal protein constituents of milk i.e. casein and lactalbumin, contain about 15.7 per cent of nitrogen.

(u) Determination of the fat content:

By the use of the soxhlet method (Hawk and Borgeim (loc cit))

Reagents:-

(1.) Ether pure

Procedure:-

Five gms. of the prepared sample after accurately weighing were introduced into the fat-free extraction thimbles, and were extracted with pure ether for 3-5 hours in a soxhlet apparatus, the extraction being carried until fresh portions of absolute ether gave no further trace of extractive material. Using a safety water-bath, the flask containing the fat was heated to a constant weight at a temperature below 100°c.

In case of samples of the articles of food which were liquid e.g. milk, Adam's paper-coil method was used. About 5 cc of milk were introduced into a small beaker and the accurate weight was quickly

ascertained. A fat-free coil was made to stand in the beaker and the coil was rotated by inclining the beaker, in order to hasten the absorp-tion of the milk. Immediately upon the complete absorption of the milk, the coil was removed and again the weight of the beaker was quickly ascertained. The difference in the weights of the beaker at the two weighings represented the quantity of the milk absorbed by the coil. The coil was dried very carefully at a temperature below 100°c and extracted it with ether. The further procedure was the same as in the case of the method for the samples of the articles of food which were solid.

In case of the articles of food which were semi-solid e.g. white of the egg, a long and a little wide strip of a fat-free filter paper was used instead of the paper coil and the accurately weighed quantity of the egg-white was spread on the strip very carefully and the strip was allowed to dry carefully at a temperature below 100°c. The further details of the procedure were the same as described above for liquid samples of food.

Finally, the fat content was calculated in all cases in terms of gm. per 100 gms. of the sample of the article of the foodstuff.

(v) Determination of the carbohydrate content:

The carbohydrate content in the articles of the foodstuff was determined by difference only i.e. the sum total of the moisture, fat, proteins and ash content was subtracted, assuming that the remaining was the carbohydrate content present in the article of the food.