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PART I.

ORIGINAL COMMUNICATIONS.

ART. XIII.—*The Modifications undergone by Fatty Complexes in the Alimentary Mucosa.*^a By F. W. LAMB, M.D.: Assistant to Professor of Physiology, Trinity College, Dublin.

I PROPOSE to examine in outline, firstly, the problems of fat absorption which have been from time to time the subject of investigation, and the methods which have been used for their elucidation; secondly, the histological methods at our disposal; and, lastly, the results which I have obtained from the application of some of these methods together with some generalisations founded thereon.

A review of the lines of experimentation which are reported in the literature shows that a classification of methods and questions at issue may be made on the following lines :—

1. In the first place, *chemical investigations*, which have largely monopolised the field, can obviously give

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information on the more general points of fat metabolism—the general characters of fatty complexes; the existence and extent of desaturation in the total fat content; various quantitative comparisons between protoplasmic and depôt fat; the distribution of fatty bodies during fat transportation, and problems of like nature.

Yet the application of purely chemical methods has afforded only comparatively meagre results when applied to fat absorption questions. For example: since Munk (1) showed from examination of the thoracic duct chyle that the intestinal tract was the site of neutral fat synthesis, and Moore and Rookwood (2) approached the site of the process in the intestinal epithelium by an examination of the chyle in the mesenteric lacteals, little was done to investigate the more intimate cellular phenomena until the publication of a paper by Noll (3).

2. On the other hand it is obvious that it is from *chemical and physical histology* we must expect results when dealing with intracellular processes.

The difficulties and fallacies of such methods are notorious, but the order of the phenomena is microscopical.

The main problems to be solved may be divided into the nature and extent of changes undergone by fatty substances in the intestinal lumen, the method of actual transport into the alimentary mucosa, the phenomena of the deposition of fatty bodies in the epithelial cells, and the relation of the different parts of the cells thereto; the transport from these cells into the lacteals, the physical and chemical properties of the fatty substances in the lacteals and in the thoracic duct.

I do not propose to refer in the present paper to several other problems which have been the subject of a certain small amount of work—for example, the changes taking place in lymph glands and the possibility of direct absorption into the blood.

The set of phenomena more immediately concerning me is that presented by the fatty intracellular globules and

the staining reactions which can be obtained from the methods at our disposal.

When we review these latter it is convenient to classify them into—(1) methods which I propose to call morphological, that is, those which afford evidence of the presence of fatty bodies in tissues, but which give slight clues to the individual constituents or to any changes which are taking place; (2) methods which afford some chemical or biochemical information.

1. In greater detail the first class, *morphological methods*, may be divided into :—

(a) Staining by the use of dyes, “insoluble” with fatty substances, applied to frozen sections. To this class belong the well-known Sudan III., and Scarlet Red dyes (Daddi, 1896; Michaelis, 1901, *vide* Mann (4)).

The oxazone dye, which is easily formed by intramolecular change from the oxazine dye, Nile Blue (*vide infra* (A) (b)), is another example of this class.

It cannot be too strongly emphasised in this connection that these stainings depend entirely on the question of solubility: the dyes do not stain fatty substances in the solid crystalline condition. It is certainly possible to get different nuances of colour staining with them in a section, but it is impossible to draw any valid conclusion therefrom, as this difference depends on degrees of fluidity or size of the globules (*vide infra* (A) (b) and Fig. 6). A simple experiment will prove the former. A fatty body, solid at ordinary temperature—*e.g.*, stearic acid—placed in contact with one of these dyes remains unstained until the temperature is raised to the melting point. Similarly, frozen oleic acid does not stain with osmium tetroxide. Pure cholesterol and pure tributyrin do not stain, but when they are mixed blackening is rapid and intense (33).

(b) The second general method of procedure is to use some fat fixative, then to embed the tissue in paraffin. The fixation may have different degrees of action on the various fatty and lipid compounds, and thus conclusions may be drawn as to the presence of certain substances

which are fixed by the reagent employed, so that they remain undissolved during the embedding procedure, and are made evident by subsequent appropriate staining. The fixative procedures that have been employed are—(1) chromation (*e.g.*, potassium dichromate), and then staining by Sudan III. Bell (6) has employed this method, and has drawn conclusions from the presence of a “Sudanophil” precipitate regarding the occurrence of “lipoid” (*cf.* also Ciaccio 7 and 8). (2) Osmication. Osmic acid, as is well known, renders certain substances insoluble in fat solvents, with a consequent resistance to the solvent action of the paraffin procedure.

This osmication can be direct (9) or followed by alcoholic treatment (10). The latter produces a further reduction where the sole application of osmic acid produces little effect.

(c) Observations can be made on the arrangement and form of the vacuolation, in stained paraffin sections. Stewart (11) has used this method in order to observe the effect on tissue elements from the deposition of cholesterol crystals. This procedure has the further merit of allowing observations on tissue changes—*e.g.*, the extent of leucocytic invasion, cell proliferation, or other changes accompanying fat deposition.

2. Procedures which allow of more or less certain deductions regarding the chemical nature of the fatty complexes. We may refer to them as *chemical and biochemical methods*. It is, however, to be borne in mind that the term chemical is a too narrow one. The physical conditions are of equal or perhaps of greater importance. The transport of fat from the intestinal lumen to epithelial cell is essentially one of relative solubility (partition) in the intestinal contents and in the epithelial protoplasm. This was overlooked in many of the old discussions on the necessity for soap formation as an antecedent to absorption (24). Further, no fatty globules in tissues are chemically pure—the admixture of minute amounts of lipoids with absorbed fat is of great importance. Again, the question

of the conditions which give rise to histological "masking" of tissue fat are probably physical rather than chemical in nature (12).

The methods here referred to may be divided into (A) a general group which affords some evidence of chemical properties, and (B) a group which, depending for its results on a progressive oxidation procedure, merits special attention.

(A) Taking the more general group first, let us see what chemical properties the fats and lipoids present which may form the basis of methodical investigation.

(a) "*Unsaturation*."—The methods available are the osmic acid (Altmann, 1894 (9), or chromation, with subsequent hæmatoxylin staining; that is the production of a chrome hæmatoxylin lake fixated to the unsaturated linkage (Lorrain Smith and Mair, 1908) (18). As all fatty complexes in the tissue contain unsaturated members, the mere observation of unsaturation of itself is not of great interest, except in the case of the absorption of a saturated fat (*vide in frà*). No valid deductions of the amount of unsaturation can be drawn from the degree of osmication, for osmic acid readily yields up its oxygen. This liability in fact limits to a certain extent its usefulness. Further, the physical conditions are of importance. For example, in Fig. 6, the region enclosed in a circle contains a deposit of lipid globules, which contain many unsaturated groups in fluid crystalline condition, which are only slightly browned by osmic acid, while in the same neighbourhood can be seen fat cells, whose contents take on a deep black colour. The conditions are probably similar to those which produce difference of colour with intersoluble dyes.

(b) *Neutrality*.—The Nile Blue sulphate method of Lorrain Smith (5) affords a histological method, which, subject to a certain reservation, allows of observation on this point. The reservation is, that while the red colouring matter (an oxazone) belongs to the group of intersoluble dyes, therefore rapidly staining all liquid fatty

globules, whether neutral or with free acidic members, the formation of a coloured soap by the Nile Blue base (an oxazine) is a slightly slower process, therefore it is necessary to wait for this to take place before drawing conclusions, and at the same time to guard against the hydrolytic effect of CO_2 on the neutral fats (*cf.*, section 1, on neutrality).

(c) *Presence of Soaps.*—It has been claimed that the use of Fleming's solution will detect the presence of soaps, but I have never been able to convince myself of their occurrence in normal tissues nor of the reliability of the methods proposed.

The histochemical methods for detecting the presence of soaps require more extended examination before many statements in the literature can be received.

The cuticular zone of the epithelium is a place where, if anywhere under normal conditions, the presence of soaps might be expected, yet I have never been able to detect here any staining phenomena which might point to their presence. The fat here is "masked" in a most remarkable way.

(d) *Facility with which the globules undergo hydrolysis.*—The method (13) consists in staining with a basic aniline dye and exposing the sections placed in a layer of gum solution to the action of carbon dioxide of weak concentration. The rapidity with which a coloured soap forms indicates the facility with which neutral fat globules undergo hydrolysis. It is obvious that this method requires the strictest control by testing the sample of fat under similar conditions and by drawing conclusions from the examination of sections recently fixed in neutral formaldehyde, only where a marked difference is apparent.

(e) *Solubility in fat solvents.*—In chemical investigations the relative action of the various fat solvents has been widely utilised, and to some extent a similar procedure has been applied in histology. Noll (3), making use of this method, has come to conclusions on glyceride synthesis

similar to those which I have formed by the employment of the chrome hæmatoxylin process (*vide infra*).

The staining procedure may be :—(1) Osmication, with subsequent application of the fat solvent ; (2) or the staining with Sudan III. or similar dye, of sections which have previously been treated with a given solvent.

The process can be modified by using a fat solvent which has been previously saturated with a given fat or lipid, but its application is limited in view of the phenomena of intersolubility exhibited by the bodies under discussion.

(f) *Polarisation phenomena*.—In the case of lipoidal mixtures which form anisotropic globules between certain ranges of temperature, the polarisation microscope can be used to determine two points—viz., if anisotropic globules are laid down in the cells, and, if present, whether they present the same “clearing point” as that of the mixture given in the food (14).

I may remark that I have seldom met with anisotropic intracellular globules in the alimentary mucosa, especially of Reptilia. Two conditions may hinder their detection : the melting point of these bodies may impede their absorption, and the minute quantities in which they seem to be present may be insufficient to give anisotropism. I have had more success with the method in the case of warm-blooded animals. The phenomena of anisotropism, of course, is not due solely to the presence of cholesteryl-esters (14).

(g) *Staining by acid dyes*—viz., the methods of Weigert or Altmann with acid fuchsin (15). The lipoids for which this method is of use are lecithin and sphingosin, which have basic groups. Altmann's granules are considered by most recent workers to have lipoidal constituents of a phosphatide nature (16), (21).

(B) The methods which depend on the “*progressive*

oxidation'' of unsaturated linkages present very many points of interest, and have opened up many fresh paths for the elucidation of intracellular fat metabolism.

The process consists in the action of dichromate of potassium on fatty acids, fats, or lipoids, which have in their molecule one or more unsaturated linkage. At an intermediate stage the chromium compound is fixed to these links, and so serves to anchor the hæmatoxylin, so that the chrome-hæmatoxylin-fat or lipid complex resists the bleaching action of borax ferro cyanide solution or of the Pal method. That the method stains only unsaturated groups cannot be too strongly emphasised in view of some erroneous statements in the literature. For example, Kawamura in his Monograph (17) has the following statements :—

“Gegen die Smithsche Methode verhält sich das Olivenöl negativ . . . Die reine Stearinsäure färbt sich bereits nach zwei Tagen schwarz.”

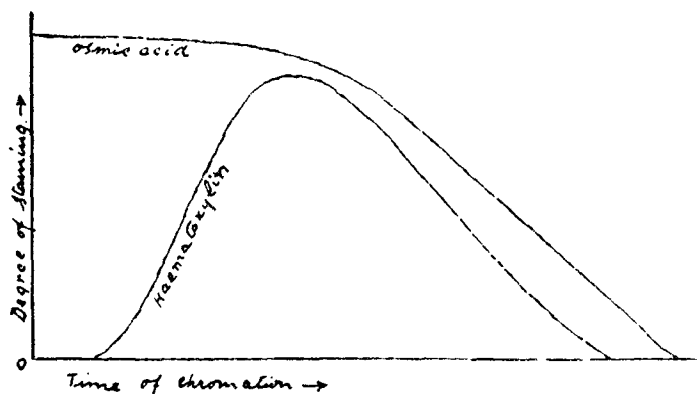
Stearic acid, being a saturated acid, never stains with this procedure, while olive oil stains at the same rate as oleic acid.

The details and theory of the method have been so fully described by Lorrain Smith and Mair (15), (18), (19) that it is unnecessary to discuss them in this place. It may be useful to emphasise how the method may be standardised and comparative results obtained (20). Frozen sections after formaldehyde fixation are placed in a large bulk of potassium dichromate solution of a given strength, and maintained at a suitable temperature.

Artificial sections—*i.e.*, the sample of fatty substances which has been fed—smeared on pieces of cigarette paper, are treated under exactly similar conditions. At 24 or 48 hour intervals, sections from both series are stained and subsequently bleached for fixed times, and the degree of

staining is observed. The stains employed subsequent to chromation may be—(a) hæmatoxylin, or (b) osmic acid.

The following diagram represents the course of the process :—



(a) It will be observed that, using hæmatoxylin, there are three stages: first, unstainable stage, when no chromium complex has yet been formed; second, a stage of gradually increasing depth of staining—this is the stage which I have found most useful; third, a stage of gradual decreasing depth of staining, followed by the final result, complete saturation (*e.g.*, oleic acid to dioxystearic acid); hence the disappearance of the chromium complex, with consequent inability of the fatty globules to stain.

(b) Where osmic acid is employed, the staining is obtained from the beginning, but gradually diminishes in intensity parallel to the disappearance of the double bonds.

The figures (1) (3) show two examples of comparative results which I have obtained, the comparison being in each case with the dépôt fat in pieces of adipose tissue in the same sections. Comparison can also be obtained within the boundaries of the same cell (*cf.* Figs. 7 and 8).

I now desire to recount the results which I have obtained by use of certain of the above methods. The animals used have been both warm and cold-blooded. There is, however, considerable difficulty in feeding warm-blooded animals on fatty substances, as it is generally impossible to give these alone, and the presence of other food complicates the results.

As the principal object was to obtain comparisons between the staining reactions of the fatty mixtures fed and those of the resulting intracellular globules, it was found that frogs were the most suitable for experiment. These animals can be used in the fasting condition. Further, by the use of liquid paraffin the alimentary tract can be emptied of its contents. It need hardly be mentioned that before valid conclusions can be drawn, the staining reactions in sections from fasting animals must be carefully investigated, and when specimens from animals in which absorption has taken place are examined, the extent and topography of the absorbed bodies must be ascertained by Sudan III. or Scarlet Red staining.

1. *Neutrality.*—In all cases (with the possible exception of the gastric mucosa) when free fatty acids or lipoids with acidic properties have been given, the resulting intracellular globules have shown a red stain with Nile Blue. In the case of fatty acids, as detailed below, the production of a triglyceride does not occur at once, yet the globules do not take a blue stain: there is no explanation of this anomaly.

2. *Fats or fatty acids of the saturated series* become capable of staining with osmic acid or with the chrome hæmatoxylin method after absorption (Fig. 12).

The staining is not as intense as in the case of unsaturated fats, yet it is quite distinct. It seems to be situated principally at the surface of the globules.

The explanation of this result may be—(1) that the absorbed substances are associated with unsaturated lipoids, present in the cell or derived with them from the intestinal contents, or (2) that the absorbed fats are themselves desaturated.

3. *Progressive oxidation methods* applied to samples fed show different rates of staining before and after absorption. Triolein is the only case where this difference is slight. The acceleration of staining after this fat has been absorbed is one to two days at room temperature with saturated dichromate.

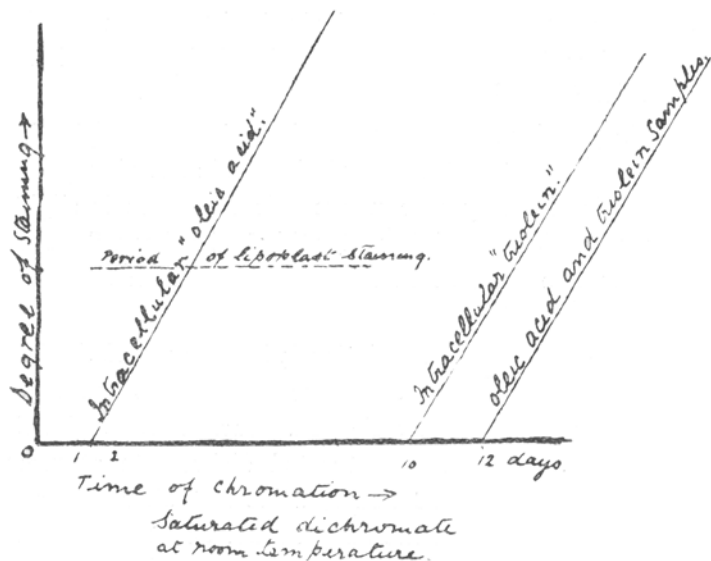
The most remarkable variation is in the case of the free fatty acids. Here the difference amounts to ten days. For example, absorbed oleic acid reaches the stainable stage when treated with saturated dichromate at room temperature in 24 to 48 hours, while free oleic acid and triolein treated outside the body cannot be stained before 12 days. They are stainable at the same rate.

Put in diagrammatic form, the phenomena can be exhibited as follows, p. 292. In other words, the absorbed acids are not immediately synthesised to the corresponding glycerides, but there is some intermediate stage in which the globules are easily oxidised. A mixture of free oleic acid and glycerol leads to the deposition of globules similar in behaviour to those appearing when triolein is fed.

Noll (3) came to the conclusion that such an intermediate stage existed. The methods that he used did not afford him an opportunity of observing the acceleration effect. The difference in staining rate can be observed to persist in fat that has passed into the lacteals.

A further detail can be made out. In the case of triolein or oleic acid fed with free glycerol, there is a stage obtainable when the intracellular globules are not yet stainable, but situated between them there are bodies stainable at a

very early stage. They are best obtained by the action of 1 per cent. dichromate at 37° C.



This is indicated in the diagram. The appearance of these bodies, which may be named "lipoplasts," can be seen in Figures 7 and 8 (32). Thus, a differentiation may be made within the limits of one cell as to its fatty constituents (21) (33).

In the case of oleic acid absorption, the stainable stage is reached so soon that it is impossible to observe whether such bodies are present separated from fatty globules.

It might be suggested that they are fused with the globules, and to this fusion is due the early staining, while, when synthesis is complete, these lipoplasts are segregated from the triglyceride, and the latter is now practically *dépôt fat*. It is interesting to note that the lipoids of the adrenal cortex are easily stained. The function put forward for this part of the adrenal is lipoid elaboration (25). What body plays the chief rôle in these phenomena? Elsewhere I have suggested cholesterol (20), (31).

The evidence for this view is—firstly, mixtures contain-

ing cholesterol are easily stained; secondly, cholesterol is able to antagonise the irritating effects of free fatty acids (*cf.* Fig. 10). I have reported elsewhere this action of cholesterol (22) (*cf.* 23, 25).

As to the source of the cholesterol, it is known as a universal constituent of protoplasm, so that it may be present in the cell, or it may come from the bile. The latter source is supported by the observation that oleic acid in the intestinal contents is already easily stainable.

4. *Anisotropic lipoids* and lipoidal mixture are rarely detectable as such after absorption. I have already referred to this. It is further evidence of the modifying influence in the intestinal mucosa.

5. The morphological pictures are different in regard to the *type of vacuolation*. This can be easily seen in paraffin sections (*vide* Figs. 9, 10, and 11).

This difference can also be seen in frozen sections treated by the hæmatoxylin method (*vide* Figs. 4 and 5). Other points can be made out from inspection of the intestines, where fat absorption is going on, and from paraffin sections. As already noted, free fatty acids have an irritating effect, and may even cause the death of an animal. The intestines show injection of the capillaries and epithelial desquamation (Fig. 10). This is an extreme stage. With the feeding of other mixtures varying degrees of vascular reaction can be seen, both as regards the extent of leucocytic emigration (Fig. 11) (varying chemotactic influence?) and as regards the degree of the vascular dilatation.

6. *Fat absorption in the stomach* presents several points of interest, to which I drew attention some years ago (20).

Subsequently other observers investigated this site of absorption (26), (27), (28).

The figures 1 and 2 show the histological picture that can be obtained. The special points which I have observed are—(a) the fat, both intra-epithelial and in the sub-mucosa, is as easily stainable by the chrome hæmatoxylin method as that in the adrenal cortex. It is, therefore, quickly oxidised.

(In the case of the fasting frog there is a continuous row of globules staining with fat stains below the free border of the epithelial cells on the surface. These may modify the reactions of absorbed fat, but in the case of warm-blooded animals I have not been able to observe this appearance.)

This facility of staining cannot be due to cholesterol from the bile, as these phenomena can best be observed in the region of the cardia. It may be due to the absorption by the stomach of fats which are easily oxidised by the method or to a mixture with cholesterol which has been supplied by the cells. (b) The staining with basic aniline dyes is more rapid than the staining of fat in the intestinal mucosa from the same animal which has been fed on a mixture of fat, such as occurs in milk. In other words, it is more easily hydrolysed under like conditions. Nile Blue stains the globules a neutral tint.

(c) In the same animal the amount of fat in the gastric mucosa is in inverse proportion to that in the intestinal mucosa. Obviously, the stages of absorption in the stomach precede these in the intestines.

It is hard to conceive that soap formation can take place. I am inclined to the view that the epithelial protoplasm has a solvent affinity for at least certain fats or fatty acids. This is supported by cases where I have found some evidence of fat absorption in the frog's œsophagus. It is also in harmony with the slight absorption which is stated to take place in the intestines after ligation of the bile and pancreatic ducts.

7. *The influence of the nucleus of the cell on fat deposition* (29). It is difficult to decide if such an influence exists. I have noticed in the case of lower members of the fatty series that the side of the cell next the intestinal lumen tends to be free from globules, which increase in number in the nuclear region. In Fig. 12 it can be seen as a fairly broad zone, free from fat.

8. *The importance of triolein or oleic acid.* I have been often struck with the difficulty of obtaining absorption with

fat mixtures which do not contain triolein or oleic acid. The glycerides of the lower saturated fats, though liquid at ordinary temperature, are not absorbed easily.

I cannot say whether saturation of the carbon chains has anything to do with this. It may be that triolein and oleic acid are especially soluble in protoplasm or that fatty acids of the unsaturated series are easiest of absorption.

9. I have endeavoured to find if the bodies which I have named lipoplasts have a relation to *Altman's granules*, but hitherto I have failed. In fact, so far as my observations go, it would seem that the Altmann bodies disappear where fat absorption is taking place.

The main conclusion which I would draw is that the *alimentary epithelium is the primary site of fat specialisation*. Although fat foreign to the animal's body can be laid down in fat depôts, yet the vital activities of the cell endeavour either to absorb fatty mixtures which resemble the fat peculiar to the species or else to produce transformations which lead to this specialisation (30).

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EXPLANATION OF PLATES.

1. Stomach, kitten : frozen section. Saturated potassium dichromate at room temperature for one day : $\times 80$. Depôt fat in mesentery unstained.

2. Stomach, cat : frozen section. Saturated dichromate at room temperature for one day : $\times 60$. From a drawing. Fat in epithelium and in submucosa stained. Depôt fat unstained.

3. Adrenal, cat : frozen section. Saturated dichromate at room temperature for one day : $\times 80$. " Fat " in cortex stained. Depôt fat unstained.

4. Intestine, frog : oleic acid and cholesterol feeding. Saturated dichromate at room temperature for three days : $\times 330$.

5. Intestine, frog : oleic acid feeding : frozen section. Saturated dichromate at room temperature for three days : $\times 330$.

6. Wall of gall-bladder (cholelithiasis). Formol fixation. Frozen section. Osmic acid. $\times 300$. The cells within the circle are loaded with anisotropic lipoids but stain imperfectly. The larger blackened droplets are isotropic.

7. Intestine, frog : triolein feeding : frozen section. One per cent. dichromate at 37 C. for two days. $\times 450$. From a drawing.

8. Intestine, frog : triolein feeding : an epithelial cell. $\times 450$. One per cent dichromate at 37°C . for three days.

9. Intestine, frog : triolein feeding : paraffin section : $\times 335$. Hæmatoxylin and eosin. Shows type of vacuolation.

10. Intestine, frog : oleic acid feeding : paraffin section : $\times 335$. Hæmatoxylin and eosin. Shows desquamation of epithelium.

11. Intestine, frog : oleic acid and cholesterol feeding : paraffin section : $\times 450$. Hæmatoxylin and eosin. Shows type of vacuolation and leucocytic infiltration.

12. Intestine, frog : Tributyrin feeding. Frozen section : $\times 80$. Saturated dichromate at room temperature for four days.

ART. XIV.—*The Frequency of Thyroid Insufficiency in General Practice.* By REGINALD JOHNSON, M.D., B.A.O., B.Ch. (Univ. Dubl.); Capt. R.A.M.C.; late lecturer on Midwifery and Gynæcology to Federated Malay States Medical School, Singapore.

FOR some nine months before I took a Commission in the R.A.M.C. I practised near Birmingham amongst working men and their families. Both men and women worked extremely hard, the women in addition to long hours spent in front of the furnace had the heavy strain of frequent pregnancies; they seemed to be continually aborting or bringing forth full time children. In many cases in less than seven days after confinement one saw them again wielding the hammer and knocking the red-hot iron into the requisite shape, whilst the latest arrival lay in a convenient niche near the furnace awaiting its next feed.

Many munition and war-workers were numbered amongst my patients. Without exception these people were too busy or too much disinclined to attend to matters of general hygiene. Constipation was their greatest trouble, and if evacuation of the bowels occurred twice a week they were satisfied. Hard work, constipation, and in the case of the women, the extra strain of frequent pregnancies kept the thyroid gland working at very high pressure and under adverse conditions. By a thyroid in-