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A Simple Fluorescence Comparator and its Application to the Determination of Porphyrin

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(*Received 13 October 1942*)

Several substances of biochemical interest are either themselves fluorescent in radiation of suitable wavelength, or are readily transformed quantitatively into fluorescent materials. Among these may be mentioned the porphyrins, stercobilin and related pigments, flavins, including vitamin B_2, certain chemotherapeutic drugs and aneurin.

Quantitative methods based on the measurement of fluorescence intensity have the advantages of being highly specific and extremely sensitive, enabling very small quantities of material to be determined with accuracy once they are suitably extracted. Such methods suffer, however, from the disadvantage of requiring, for accurate results, relatively expensive apparatus such as the combination of an ultraviolet source with the Zeiss or Pulfrich photometer or some type of photoelectric colorimeter.

For the purposes of a particular investigation it became necessary to carry out a number of quantitative determinations of porphyrin in single specimens of urine, each containing about 2μg. of porphyrin. Only measurements of fluoresence intensity were sufficiently sensitive, and in order to bring the method within the reach of the small medical or factory clinic an inexpensive fluorimeter or fluorescence comparator was essential which, acting very much like an ordinary colorimeter, would give results of sufficient accuracy for general purposes. It was realized that, with suitable adjustments, the instrument would serve for the determination of a number of fluorescent materials in addition to porphyrins, e.g. vitamins, drugs etc., as noted above. Its construction, and operation with porphyrin solutions, will be described in the present paper, whilst other applications will be dealt with in subsequent communications.

Requirements of a fluorescence comparator

The essential requirements of a fluorescence comparator are:

(1) An easily operated source of ultraviolet radiation with suitable filter to remove visible light.

(2) An optical system to conduct the visible fluorescent light into the eyepiece. This should not be in the same axis as the ultraviolet source. The emergent fluorescence is collected in the present instrument at an angle of 55° to the ultraviolet beam and is reflected by a system of mirrors into the eyepiece, the optical path being approximately equal to the distance of normal vision, so that lenses may be dispensed with. A suitable filter is interposed, the transmission of which covers a suitably narrow band in the region in which the fluorescence lies. With porphyrins, Ilford spectral filter no. 607 (orange) transmitting 595–650 mμ is used.

(3) A device for altering the intensity of one beam. In the present instance glass screens made by photographing a standard neutral-tinted wedge are used, and by adopting a reciprocating arrangement, rotation of a graduated knob brings about a gradual, but uniform, darkening of the field emanating from the standard. Matching is performed as in a colorimeter.

The construction and mechanical arrangements of the apparatus are described in the Addendum by Dr E. Schuster who has been responsible for the detailed design and who has kindly constructed the first four models produced.

METHODS

Calibration and operation of the apparatus for the determination of porphyrin

The extraordinarily intense fluorescence of porphyrin solutions has been studied by a number of workers from Dhéré [1933] onwards, one of the most detailed of recent investigations being that of Fink & Hoerburger [1933] in which the effects of *p*H, salt concentration, temperature etc. were described. These authors showed that porphyrin isomers could be recognized by differences in their *p*H-fluorescence curves.

Fikentscher [1932] first described a method of porphyrin determination based upon the measurement of fluorescence intensity in a Zeiss step-photometer, and in his article will be found a consideration of the factors governing the accuracy of any such method. With concentrations of from 0 to 10 μg. porphyrin/ml. fluorescence intensity is proportional to concentration (other things such as incident illumination remaining constant), but above this limit quenching intervenes.

An attempt to reduce the cost of the necessary apparatus has been made by Stevens & Turner [1937] who describe a fluorescence method in which a carbon arc serves as the source of ultraviolet radiation, and the fluorescence elicited is measured by an electrical circuit containing two photo-cells.

A much more convenient and relatively inexpensive source of ultraviolet radiation is the 'Osira' mercury discharge lamp with Wood's glass bulb, which is incorporated in the present machine. As fluorescent standard, a solution of coproporphyrin in 5% HCl is employed in a sealed Monax glass test tube, the concentration being 2 μg./ml. This is made up from the tetramethyl ester as follows: A stock solution is first prepared by dissolving 1·085 mg. of solid coproporphyrin tetramethyl ester (equivalent to 1 mg. free coproporphyrin) in 5 ml. of glacial acetic acid, and adding 5% HCl (137 ml. conc. HCl to 1 l.) to a total volume of 100 ml. This stock solution contains 10 μg./ml. and should be kept in the dark in a stoppered bottle. The acetic acid it contains increases its stability. Solutions of lower concentration are prepared from it by dilution, as required, and in such a way that the final concentration of HCl is 0·25% (see later). Fikentscher reports that his standards (0·375–0·75 μg./ml. made up in 5% HCl without acetic acid) suffered no change in 6 weeks.

Attempts to find a stable fluorescent dye to serve as an artificial standard in place of porphyrin have not been successful. An ideal standard, in addition to possessing stability, should both absorb at the same wave-length in the ultraviolet, and fluoresce in the same spectral region, as does coproporphyrin. Several substances have been investigated, one of the most promising being resorufin (alkali salt), but the intensity of its fluorescence is such that in concentrations sufficient to match 2 μg. porphyrin/ml. quenching occurs. Safranine, the use of which was kindly suggested to me by Mr Jope of the Nuffield Laboratory, Oxford, also exhibits quenching at concentrations higher than about 30 mg./l., corresponding in fluorescence intensity to 0·3 μg. coproporphyrin/ml.

A coproporphyrin standard which has been in regular use in one apparatus in a sealed tube has shown no deterioration during nearly 3 months.

Monax glass test-tubes are used for both standard and unknown solutions. This glass does not fluoresce in ultraviolet radiation and is moderately transparent. Other suitable makes such as Hysil (for which suggestion I am indebted to Mr Jope) may also be employed.

To calibrate the instrument the standard 2 μg./ml. porphyrin solution in its sealed tube is placed in the right-hand side of the instrument and varying dilutions of porphyrin in similar test tubes in the left-hand side; sets of 5 or 10 matchings are then recorded for each dilution. With the instruments we have constructed a plot of the scale reading against the porphyrin concentration exhibits a linear relationship (see Fig. 1). Since accuracy of

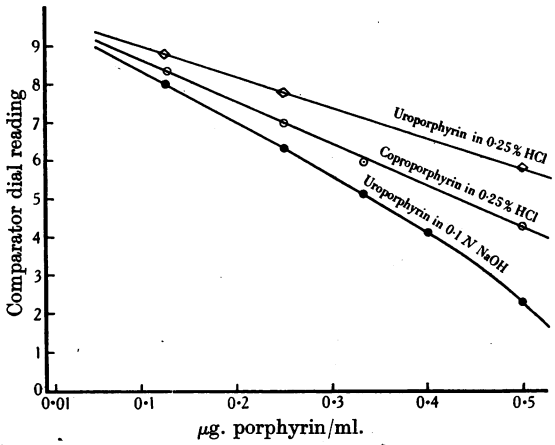

Fig. 1. Calibration curves of fluorescence comparator for porphyrin.

matching is greater at the lower intensities, it is best to dilute a more concentrated solution with 0·25% HCl until it contains about 0·25 μg./ml. which affords a convenient intensity of fluorescence. The range of the instrument may be taken as being from 0·05 to 0·5 μg. porphyrin/ml.

A porphyrin solution of unknown concentration is examined in exactly the same way, the mean of 5 or 10 matchings being accepted and the corresponding porphyrin concentration read off from the calibration graph.

As in all colorimetric work, it is advantageous to work in the dark. The sensitivity of the human eye to differences in colour intensity (especially reds) is known to vary considerably, but there is no doubt that any particular observer improves with practice.

Relation between pH and intensity of fluorescence

Fink & Hoerburger [1933] investigated the fluorescence of a variety of porphyrins both natural and synthetic, using buffer solutions to cover a *p*H range of from about 2·0–8·0, and found that, in general, fluorescence is minimal in the region of the isoelectric point, but increases progressively as the solution becomes more acid or more alkaline. An exception was found in the case of the aetioporphyrins which possess no carboxyl groups and fail to show the increase of fluorescence in alkaline solutions. The conclusion was drawn, therefore, that it is the + or − charged porphyrin molecule which is responsible for fluorescence.

As stated above Fink & Hoerburger's [1933] investigations did not extend to solutions more acid than *p*H 2 nor more alkaline than *p*H 8. Fikentscher [1932] had previously, and somewhat arbitrarily, selected 5 % HCl as the medium to be used as solvent.

Using the fluorescence comparator described in this paper and a fixed standard, it was at once apparent that fluorescence in 5 % HCl is much less than maximal and that small variations in acid concentration in this region result in relatively large discrepancies.

The *p*H fluorescence curves of coproporphyrin (I) and uroporphyrin (III) were explored from the isoelectric point to regions of high acid concentration as follows: Stock solutions of the porphyrins were made up from the esters in 5 % HCl containing 5 % acetic acid as previously described, the uroporphyrin concentration being 20 μg./ml. and the coproporphyrin 10 μg./ml. It may be noted here that 5 % HCl is the most convenient strength of acid to use in preparing solutions from the esters; the pigment dissolves in this solvent in presence of acetic acid and hydrolysis of the ester takes place slowly on standing at room temperature. Na citrate-HCl buffers [Clark, 1928] to cover the range *p*H 4·95–1·17, whilst for still more acid solutions, unbuffered HCl of concentrations 0·5, 2·5, 5 and 10 % were used. 0·1 % acid afforded a useful comparison with the buffered solutions. 0·1 ml. quantities of the stock uroporphyrin or 0·2 ml. of coproporphyrin were diluted to 5 ml. (final concentration 0·4 μg./ml.) with the appropriate buffer, the *p*H of the solution being measured by the glass electrode and its fluorescence by the comparator. In the case of uroporphyrin (III) the investigation was extended to include the region on the alkaline side of the isoelectric point by means of Na citrate-NaOH buffers. The results are shown in Figs. 2 and 3.

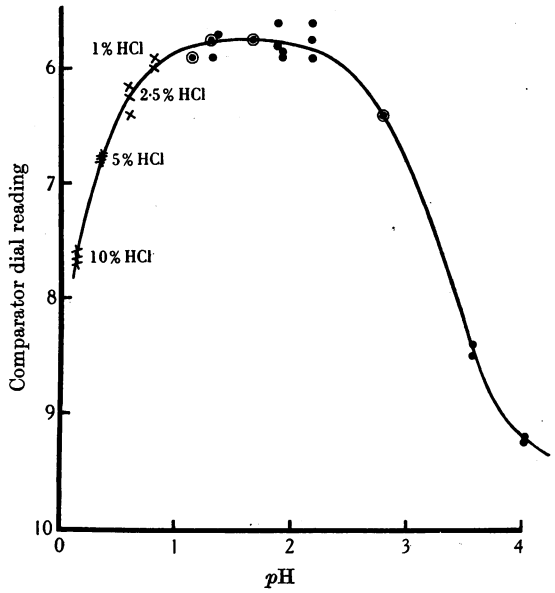

Fig. 2. Fluorescence intensity of coproporphyrin in acid solutions. Porphyrin concentration 0·4 μg./ml. • Buffered solutions. × Unbuffered solutions. ⊙ Coincident values.

The intensity of fluorescence of both porphyrins rises to a maximum at about *p*H 1·4, but declines rapidly as the concentration of acid is increased.

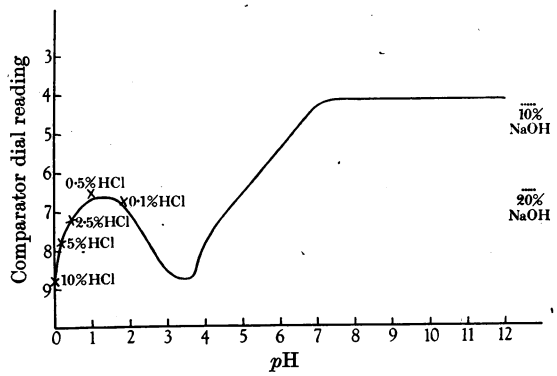

Fig. 3. Fluorescence intensity of uroporphyrin in acid and alkaline solutions. Porphyrin concentration 0·4 μg./ml.

5 % HCl is seen to be a most unsuitable solvent for quantitative measurements, whereas in the region *p*H 1·2–2·0 for coproporphyrin, or 1·0–1·6 for uroporphyrin, fluorescence intensity remains practically constant. The acid number of coproporphyrin is fortunately low, viz. 0·1, so that a solvent such as 0·25 % HCl having *p*H 1·24 can be used to extract it directly from ethereal solution.

On the alkaline side the fluorescence of uroporphyrin increases regularly from the isoelectric point to a *p*H of about 8·0 after which it remains constant until *p*H 12 (0·1 *N* NaOH) is reached. Even in *N* NaOH the intensity is substantially the same, but in 10 % NaOH it has dropped to about one-half, and in 50 % alkali it is practically extinguished.

In high concentrations of both alkali and acid, therefore, the intensity of porphyrin fluorescence is depressed. These changes are reversible, the porphyrins being relatively very stable molecules, and it seems reasonable to suggest on the basis of Fink & Hoerburger's [1933] theory that the fall in fluorescence intensity is due to suppression of the ionization of the molecule in presence of the overwhelming concentration of OH or H ions.

A practical outcome of these investigations is the suggestion that quantitative fluorescence intensity measurements for both uroporphyrin and coproporphyrin should be made in 0·25 % HCl (*p*H approx. 1·24) when small variations in *p*H will have relatively little effect upon the result. In the case of uroporphyrin it is convenient under certain circumstances to work in alkaline solution and for this purpose 0·1 *N* NaOH is a suitable solvent to employ. The fluorescence comparator has been calibrated for all these possibilities.

Equal quantities of uroporphyrin (I) and uroporphyrin (III) under the optimum conditions exhibit equal intensity of fluorescence. It should clearly be possible, however, to employ the instrument for identification of porphyrin isomers by plotting the *p*H-fluorescence curves according to Fink & Hoerburger in a series of buffer solutions on either side of the isoelectric point.

Preparation of the porphyrin extract from urine

In this paper the manipulation of the fluorescence comparator has been described first, since it is desired to emphasize that its function is to record the concentration of any given solution. Errors introduced in the preparation of the extract, variations in porphyrin excretion during the day etc., must not be confused with those inherent in the use of the comparator.

Little is known concerning the level of porphyrin excretion throughout the day, hence wherever possible a 24-hr. specimen should be collected and an aliquot portion of 25–50 ml. taken from this for analysis.* One-tenth of the volume of glacial acetic acid is then added, followed by 2 vol. of solvent prepared by mixing 9 vol. of ether with 1 vol. of light petroleum (B.P. 40–60°) and the mixture is shaken in a stoppered separatory funnel for about 1 min. The addition of petroleum minimizes the risk of emulsification whilst having no appreciable effect upon the extraction of the porphyrin. When separation is complete the lower layer is drawn off and discarded, and the ethereal layer is washed three times in succession with about an equal volume of distilled water. The porphyrin is now transferred to 0·25 % HCl by adding 2 ml. of acid, shaking and transferring carefully to a Monax test tube graduated at 5 and 10 ml., then repeating the extraction with 1·5 ml. and lastly with 1 ml. of acid. The pooled acid extracts are then made up to the 5 ml. mark, the tube stoppered and the contents mixed. It is then placed in the left-hand side of the fluorescence comparator and a set of readings made. Reference to the calibration graph gives the concentration of porphyrin/ml. of extract. Should the solution require further dilution, it may be made up to the 10 ml. mark with 0·25 % HCl. From start to finish a complete determination can be made in about 15 min. The used solvent may be collected for eventual recovery.

It will be noticed that only a single extraction of the urine with ether is performed. This is justifiable for a clinical method on grounds of speed, convenience, economy and the degree of complete extraction obtained. To perform a second extraction would mean the use of a second separatory funnel or an intermediary vessel to store the ether, and the use of twice the amount of solvent. Using solutions of pure coproporphyrin containing from 0·05 to 0·5 μg.

Table 1. *Comparison of porphyrin determinations on pathological urines by the standard method and the fluorescence comparator method*

Case	Diagnosis	Standard method μg. porphyrin/l.	Fluorescence comparator μg. porphyrin/l.	Difference %
Th.	Toxic jaundice	79·3	75	− 5·4
		89·7*		−18·5
Shep.	,,	319*	320	+ 0·3
Do.	Sprue	102	100	− 2·0
		25·2	25	− 0·8
Co.	Aplastic anaemia	70·8*	60	−15·3
Sh.	,,	111*	70	−36·9†
Sh.	,,	58*	60	+ 3·5
V.d.S.	Hepatic insufficiency	156	167	+ 7·1
		70	77	+10·0
Sm.	Hepatic cirrhosis	47·8	43	−10·0
Thom.	Addison's disease	77·7	77	− 0·9
Ber.	Acute idiop. porphyria	313	306	− 2·2
Ber.	,,	769	750	− 2·5
Ber.	,,	916	1080	+17·9
Res.	Pellagra	892·5	768	−13·9

* Mr Jope of the Nuffield Laboratory, Oxford, has informed me in a private communication that he finds the urinary porphyrin excretion may vary widely throughout the course of the 24 hr.

* Determinations made by Dr J. Kench, Royal Infirmary, Manchester.

† The acid extract was much coloured by foreign pigment (from a highly pigmented urine) which probably led to a high spectrophotometric result.

of porphyrin/ml., i.e. from approximately the normal to ten times the normal concentration in urine, and subjecting them to the extraction procedure outlined above, it has been found that the recovery is about 90 %. Should turbidity affect the final acid extract, it may be centrifuged.

Agreement with the standard procedure

In order to test the reliability of the fluorescence method when applied to normal and pathological urines, a number of specimens were analysed by this technique and also by the standard procedure of Fischer, in which to 1 l. or more of urine, one-tenth the volume of acetic acid is added and the mixture then shaken twice with at least an equal quantity of ether. After washing repeatedly with water, the ether layer is shaken with successive quantities of 5 % HCl and the porphyrin in the combined extracts determined spectrophotometrically (Hilger-Nutting apparatus calibrated by means of standard coproporphyrin solutions, readings being taken at a wave-length of 546–549 mμ in the centre of the β band of the acid absorption spectrum). Frequently, in some pathological urines, the initial acid extract contained brownish pigments without characteristic absorption spectra, which led to erroneously high porphyrin figures, but by careful repetition of the purification over ether and re-extraction with acid [Rimington & Hemmings, 1939] these could usually be eliminated and a reliable result be obtained. Through the kindness of Dr J. E. Kench of the Manchester Royal Infirmary many of the standard analyses were performed in Manchester, whilst the corresponding fluorescence measurements were made at Hampstead. Table 1 presents the results obtained.

ADDENDUM. By E. SCHUSTER

Construction of the apparatus

The apparatus is shown semi-diagrammatically in Figs. 4 and 5 which give respectively a sectional elevation and a sectional plan. It consists of a

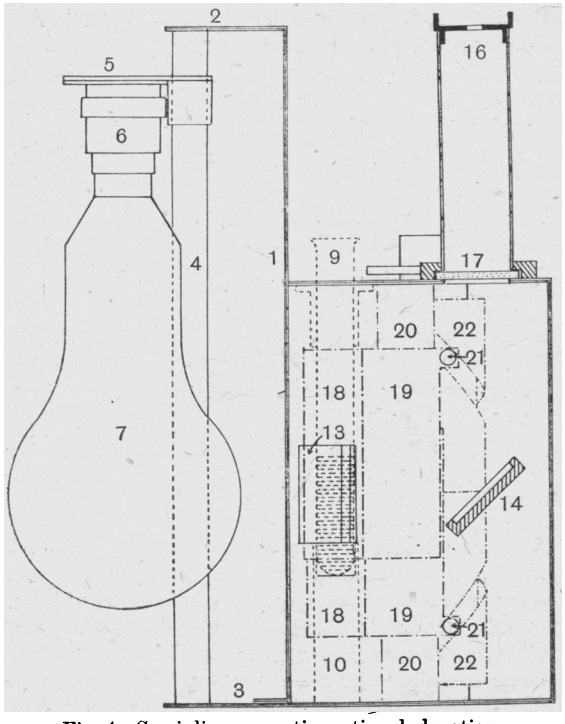

Fig. 4. Semi-diagrammatic sectional elevation (1/3 natural size).

metal box, the back wall of which (1) is raised upwards for 4 in. and then bent backwards for 2 in. to form a flange (2) which coincides in plan with a backward prolongation of the base (3). Two vertical brass rods (4) connect the base with the flange, and a horizontal bracket (5) is clamped to these rods at an adjustable level. The lampholder (6) for the Osira lamp (7) is screwed to the under-surface of the bracket. Piercing the top of the box near the back wall are two guide tubes, of which the right-hand one receives the test tube (8) containing the

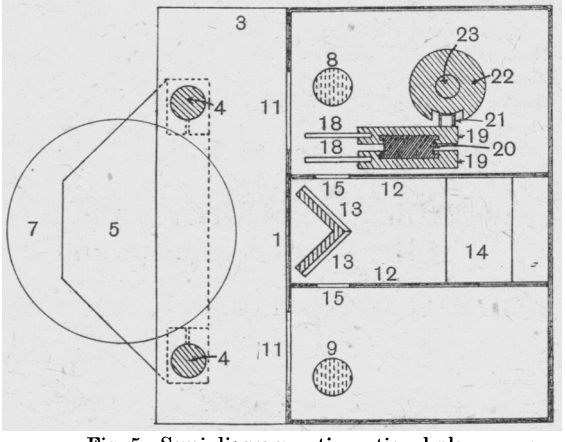

Fig. 5. Semi-diagrammatic sectional plan (1/3 natural size).

standard solution and the left-hand one the sample (9). The bottoms of the test tubes are supported by sockets (10) attached to the base. Ultraviolet rays reach a section of each test tube through windows (11) in the back wall. Small brackets surrounding these windows serve to carry extra pieces of Wood's glass, for use in determinations in which a spectral filter is omitted. They eliminate the last traces of

visible light from the source. Vertical partitions (12) separate off a central compartment of the box and serve as supports for the three mirrors which it contains. Two of these (13) lie at right angles to each other in vertical planes, while the third (14) makes an angle of 45° with the horizontal.

Fluorescent light from the two test tubes enters the central compartment through square apertures (15); it is reflected by the vertical mirrors on to the oblique mirror and thence upwards into the eyepiece (16) through a hole in the top of the box. The base of the eyepiece tube contains a filter (17) which cuts off practically all light except the wave-lengths produced by the fluorescence of porphyrin.

Interposed between the standard and the aperture (15) are the two glass screens (18); the dark end of one is above and of the other below, so that where they overlap they produce a uniformly shaded field. The screens are mounted in slides (19) which can move up or down a vertical guide (20). Each is held at the desired level by a pin (21) which enters a spiral groove in a vertical cylinder (22). The spiral is in one case right-handed and in the other left-handed. The two cylinders are fixed one above the other to a spindle (23) carried in bearings in the top and base of the box; a knurled head and a dial divided into a hundred divisions are mounted on the spindle above the top of the box. The spiral groove is of such a pitch that the whole range of movement of the slides is achieved by slightly less than one complete revolution of the spindle, and, as the grooves are right- and left-handed, equal and opposite movements are given to the two slides by a given degree of rotation.

The slides are of such a length that their less dense ends are separated by a gap of about $\frac{1}{4}$ in. when the dial knob is at zero, so permitting unobstructed light to pass in this position from the right-hand tube to the mirror for comparison with the tube on the left. This arrangement has been found convenient for certain determinations in which a blank had to be evaluated.

The inside metal work of the apparatus is painted black and the tube of the eyepiece is lined internally with matt surfaced black paper.

Thanks are due to Mr J. Smiles and his assistants of this Institute for kindly preparing the glass screens used in the construction of the instrument. I also wish to acknowledge the benefit of friendly discussions with my colleagues, Dr J. E. Kench of the Royal Infirmary, Manchester and Mr E. M. Jope of the Radcliffe Infirmary, Oxford. Dr Z. A. Leitner kindly secured some of the pathological urines used for test studies.

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The Utilization of Urea in the Bovine Rumen. 1. Methods of Analysis of the Rumen Ingesta and Preliminary Experiments *in vivo*

By R. M. PEARSON and J. A. B. SMITH, *From the Hannah Dairy Research Institute, Kirkhill, Ayr*

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Some evidence has accumulated during the past 40 years to suggest that ruminants can utilize simple non-protein nitrogenous compounds such as urea when these compounds replace a proportion of the protein normally present in the diet. The work, much of which is highly controversial, has been reviewed recently by Krebs [1937], and also by Owen [1941]. Since these reviews appeared, publications by Owen, Smith & Wright [1941; 1943] in this country and by Hart, Bohstedt, Deobald & Wegner [1939] in America have shown that urea undoubtedly has some definite nutritive value when fed to ruminants in suitable amounts and in appropriate mixtures. It has been suggested that certain micro-organisms which multiply in the rumen build up their own body protein from this simple form of nitrogen. These micro-organisms then pass further along the alimentary tract where their protein is digested with the ordinary protein of the diet. The work here described was undertaken to test the validity of this theory.

A steer with a large rumen fistula was employed and in the original plans, made in 1938, it was hoped to use this animal, in the first place for *in*