Inositol in Seminal Plasma

By E. F. HARTREE
A.R.C. Unit of Reproductive Physiology and Biochemistry, Molteno Institute, University of Cambridge

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Inositol (*meso*inositol or *myo*inositol) occurs very widely in plants, animals and micro-organisms (Fleury & Balatre, 1947; Courtois, 1951; Hawthorne & Chargaff, 1954; Mann, 1954*a*). It occurs in the free state as well as in the form of inositol phosphatides, while in plants it is often present as the hexaphosphate, phytic acid. Treatment of liver and nervous tissue by the standard procedure of Schmidt & Thannhauser (1945) gives ribonucleic acid (RNA) fractions contaminated by materials which yield inositol phosphates on hydrolysis. According to Hutchison *et al.* (1956) these consist of peptide-inositol phosphates derived from protein-bound inositol phosphates. On hydrolysis of such complexes inositol is usually liberated as the monophosphate, but Folch's (1952) analysis of neurokeratin from ox-brain showed that the inositol was present mainly as a diphosphate forming part of a lipoprotein. Of apparently more restricted distribution are free inositol monophosphate which has been isolated from guinea-pig liver (Hawthorne & Hübscher, 1956) and an inositol galactoside which has been obtained from sugar-beet molasses (Brown & Serro, 1953).

The inositol contents of the majority of plant and animal tissues, estimated after hydrolysis, vary between 10 and 100 mg./100 g. fresh weight (Mann, 1954*a*). An exceptionally rich source is the seminal vesicle secretion of the boar, in which Mann (1951, 1954*a*) found inositol levels of about 2400 mg./100 ml. In this case the inositol is present largely, if not entirely, in the free state. The present survey of the inositol content of semen and of secretions from the accessory organs of the male reproductive tract was undertaken as an introduction to the wider problems of the function and metabolism of inositol in the male reproductive organs.

MATERIALS

Semen of boar, bull, rabbit, ram and stallion was collected by means of the artificial vagina (Walton, 1945), and was supplied by the Animal Research Station, Huntingdon Road, Cambridge. Human semen from donors at the London Fertility Clinic was made available through the kindness of Dr H. Davidson. Except for the samples that are specified below, the semen was centrifuged within 2 hr. of collection in an angle head at 15 000 *g* for 20 min. The resulting clear plasma was preserved at −15° if not immediately analysed. With ram semen the packed spermatozoa in the centrifuge tube were washed with Ca-free Ringer solution (Mann, 1946). Some semen samples (nos. 16–19, 23, 51, 52) were stored at −15° as whole semen. After thawing, the plasma was obtained as above.

The materials listed in Table 3 were obtained immediately after the death of the animals. Epididymal semen, seminal vesicle secretion and ampullar secretion were expressed from the organs, the seminal plasma being separated as before. The hedgehog prostates were ground with an equal weight of water, deproteinized with 5 vol. of ethanol and the clear fluid concentrated so that 1 ml. was equivalent to 1 g. of tissue.

METHODS

Microbiological assay of inositol. The technique adopted was that of Campling & Nixon (1954), which utilizes the yeast *Kloeckera brevis* (National Collection of Yeast Cultures no. 328). The samples to be analysed were added to 2·5 ml. of inositol-free medium in screw-stoppered bottles, diluted to 5 ml. with water, sterilized for 20 min. at 120° and inoculated with one drop of a very dilute *K. brevis* suspension. Duplicate sets of standards (0–5 μg. of inositol) were set up on each occasion to provide data for a standard curve relating turbidity to inositol content. After 65–70 hr. incubation at 25° the growth was estimated by a Hilger absorptiometer with red filters (transmission peak: 675 mμ) or with an EEL nephelometer. No growth occurred when the concentration of inositol was less than 0·1 μg./5 ml. (Fig. 1). For estimations within the range

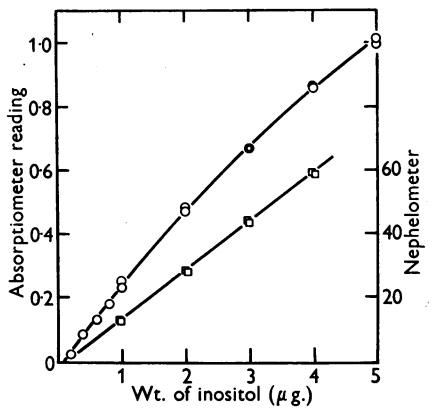

Fig. 1. Relationship between growth of *K. brevis* and concentration of inositol. Turbidity measured in the Spekker absorptiometer with red filters (○) or in the EEL nephelometer (□).

0–1 μg. of inositol the sensitivity of the method was increased by measuring growth in terms of the extinction at 800 mμ of the yeast suspension in 2 cm. spectrophotometer cells. If red light is used the slight brown colour of some hydrolysed samples does not interfere. The standard deviation did not exceed 4%.

Free inositol. The most reliable method for deproteinization of seminal plasma and of the various secretions that were investigated proved to be that developed by Somogyi (1945) for blood. To 0·5 ml. of seminal plasma was added 7·5 ml. of water and 1·0 ml. of 0·15M-$Ba(OH)_2$. After mixing, the solution was treated with 1·0 ml. of exactly equivalent $ZnSO_4$ solution. (Equivalence is determined by titrating the zinc solution, considerably diluted with water, with the baryta in the presence of phenolphthalein.) After vigorous shaking the mixture was frozen at −15° overnight, thawed and centrifuged. The freezing and thawing are not part of Somogyi's method and is unnecessary for deproteinizing blood. In the present work, however, omission of this step sometimes resulted in slightly opalescent solutions.

Total inositol. Samples were hydrolysed by refluxing for 16 hr. in HCl (reagent grade). The final concentration of acid was 6N. As a rule 0·2 ml. of seminal plasma (or other secretion), alternatively 10–20 mg. of dry material, was diluted to a final volume of 5·5 ml. and boiled in test tubes fitted with ground-in air condensers. In some cases, especially boar seminal plasma, the solution becomes very dark. Furthermore, sufficient inorganic phosphate may be released to give rise to a precipitate of heavy-metal phosphates when the hydrolysate is added to the culture medium. The following procedure reduced the colour and the phosphate concentration to a negligible level.

The hydrolysate was evaporated to dryness on a steam bath, treated with 5 ml. of water and 50 mg. of talc (British Drug Houses Ltd.) previously washed with hot 6N-HCl, and evaporated again to dryness. The cooled residue was stirred with 5 ml. of $Ca(HCO_3)_2$ solution. This was prepared by saturating an aqueous suspension of $CaCO_3$ with CO_2 for 1 hr. at 0° and allowing excess of $CaCO_3$ to settle. The solution was stored at 5°. The bicarbonate-treated residue was slowly heated to 100° and then evaporated to dryness. The residue was suspended in a known volume (usually 10 ml.) of water and centrifuged.

Known amounts of inositol added to plasma before deproteinization were recovered completely. Similar experiments using the procedure for total inositol indicated a recovery of at least 96%.

RESULTS

Specificity of meso*inositol in the microbiological assay with* Kloeckera brevis

Woolley (1941*a*) found that with *Saccharomyces cerevisiae* the microbiological method was highly specific for *meso*inositol, the isomeric cyclitols and their ethers being virtually inactive. The inositol-requiring mutants of *Neurospora crassa* can also be used for the assay (Beadle, 1944).

The use of *K. brevis* for the estimation of inositol was proposed by Burkholder, McVeigh & Mayer (1944) and the technique has been developed by Northam & Norris (1952). Although 3 days' incubation are required for maximal growth of this yeast instead of 16 hr. with *Saccharomyces* spp. it has two important advantages over the latter. *K. brevis* will not proliferate in absence of inositol and its growth is not inhibited by choline (Lovern & Olley, 1953).

The high specificity of *meso*inositol shown by *K. brevis* is illustrated in Table 1. The results were obtained by comparing the growth response to 1, 0·2 and 0·04 mg. of the substances listed with the growth response to 2 μg. of *meso*inositol.

Conditions for complete hydrolysis of bound inositol by acid hydrolysis

Preliminary experiments were carried out with ram seminal plasma in which it was found that only a small proportion of the inositol was present in the free state. While assays of free inositol yielded concordant results, estimations of total inositol were

Table 1. *Specificity of* meso*inositol as growth factor for* Kloeckera brevis *in a medium containing glucose and asparagine as carbon sources*

		Relative activity
a	*meso*Inositol	100
	*epi*Inositol	3
	(−)-Inositol	0·2
	Scyllo*meso*inosose	4
	Quebrachitol	0·5
	(−)-Virburnitol	4
	Inositol monophosphate: probably the 5-isomer (Hawthorne, 1955)	9
b	Inactive (<0·04% of activity of *meso*inositol)	

Arabinose	Dulcitol
Cellobiose	Erythrol
Fructose	Mannitol
Fucose	Pentaerythritol
Galactose	(+)-Quercitol
Lactose	Sorbitol
Maltose	Scyllitol
Mannose	
Xylose	

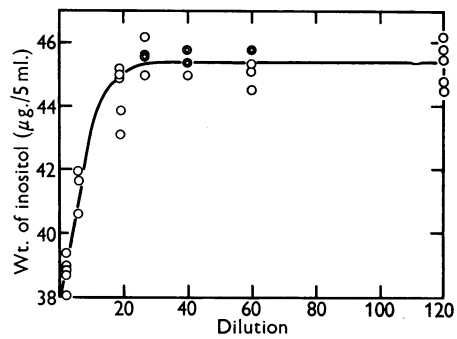

Fig. 2. Liberation of inositol by hydrolysis of ram seminal plasma in boiling 6N-HCl for 16 hr. Effect of dilution of plasma in a total volume of 5·5 ml.

not reproducible. This lack of reproducibility was traced to variations in the dilution of seminal plasma in the hydrolysis mixture, an effect that does not appear to have been recorded previously. Thus 16 hr. boiling of 5·5 ml. of 6N-HCl containing 1 ml. of plasma yielded proportionately less free inositol than did a parallel experiment with 0·2 ml. of plasma. It was found that maximal liberation of inositol from both ram and boar seminal plasma took place only when the dilution of seminal plasma was greater than 25 (Fig. 2).

The liberation of inositol from lipids of brain (Folch & Woolley, 1942) and haddock flesh (Lovern & Olley, 1953) is reported to be complete after 6 hr. boiling with 6N-HCl. Since no information is available on the nature of the bound inositol in the materials now being reported on, the effect of time of hydrolysis was determined. In the case of ram and boar seminal plasma complete hydrolysis requires 16 hr. (at a dilution of 25) although 95–97 % of the bound inositol is liberated during the first 6 hr.

The inositol level, after hydrolysis, in a mixture of seminal plasma and inositol was, in several experiments, equal to the sum of the separately determined inositol in the two components. A similar agreement in levels of free inositol was observed if the standard deproteinization procedure was substituted for hydrolysis. Thus seminal plasma is devoid of any inhibitors of the growth of *K. brevis*.

Reaction of inositol phosphatides to the standard procedures for hydrolysis and deproteinization

On the assumption that the bound inositol in the samples under examination was largely lipid inositol, tests were carried out to determine whether inositol lipids would be co-precipitated with proteins during deproteinization with the Ba–Zn reagent. A sample of mixed serine and inositol phosphatides, obtained from ox-liver, was supplied by Dr J. N. Hawthorne.

Complete hydrolysis of 1 mg. of mixed phosphatides under the standard conditions required 16 hr., the yield of free inositol being 110 μg. The assay of 1 mg. of untreated phosphatide elicited a growth equivalent to 2·8 μg. of inositol. Thus the inositol equivalent of inositol phosphatide is only 2·5 % of that of the hydrolysed phosphatide. It is very unlikely that the phosphatide contains free inositol, but slight hydrolysis probably occurred during sterilization at 120°. If the mixed phosphatides were subjected to the deproteinization procedure with $Ba(OH)_2$ and $ZnSO_4$ and the clear solution was subsequently hydrolysed, free inositol could be detected. In such experiments 10 mg. of phosphatide (containing 1100 μg. of bound inositol) yielded 184 μg. of free inositol. Thus 17 % of the inositol phosphatide was not precipitated by the barium–zinc reagents. However, such incomplete precipitation would introduce a negligible error in estimation of free inositol, since the inositol equivalent of unhydrolysed inositol phosphatide is only 2·5 %. In fact, the addition of inositol phosphatide to seminal plasma does not affect the level of free inositol determined after deproteinization. Thus 0·5 ml. of stallion seminal plasma (Table 2, sample 13), and 0·5 ml. of the same plasma in which 2 mg. of mixed phosphatides had been dissolved, were each deproteinized and samples of the clear solutions subsequently hydrolysed. Before hydrolysis the free inositol assays were 198 and 200 μg. respectively, while after hydrolysis the inositol contents were 197 and 235 μg. respectively. The production of 35 μg. excess of inositol on hydrolysis of the deproteinized mixture of plasma and 2 mg. of mixed phosphatides again indicates that about 17 % of the inositol phosphatide was not precipitated during deproteinization. However, the effect of the remaining phosphatide in the estimation of free inositol was negligible.

It was suggested (Mann, 1954*a*) that seminal vesicle inositol may derive from a phosphorylated precursor and more recently free inositol monophosphate has been detected in liver (Hawthorne & Hübscher, 1956). However, Hutchison *et al.* (1956) found that bull and sea-urchin sperm was unique, among a range of cells and tissues, in yielding 'RNA fractions' devoid of inositol phosphates. The barium salts of phosphorylated inositols containing more than one phosphate group are insoluble in water while those of the monophosphates require alcohol for their precipitation (Posternak & Posternak, 1929). Thus inositol monophosphates might conceivably be present in the protein-free filtrates after deproteinization. A sample of the monophosphate obtained from phytic acid was made available by Dr J. N. Hawthorne. This substance is probably the 5-phosphate (Hawthorne & Chargaff, 1954; Hawthorne, 1955). The assay of inositol monophosphate (containing 1 mg. of bound inositol) gave an inositol equivalent of 90 μg. When the monophosphate was subjected to deproteinization the equivalent fell to 7·5 μg., while deproteinization followed by hydrolysis gave an equivalent of 60 μg. Thus although about 6 % of inositol monophosphate remains in solution after barium–zinc treatment its effect upon the assay of free inositol after deproteinization would be negligible, i.e. less than 1 % of the effect detectable after hydrolysis.

Distribution of inositol

Inositol has been detected in every sample of seminal plasma which has so far been examined (Table 2). While the total inositol content of boar seminal plasma normally exceeds 600 mg./100 ml., that of bull, human, ram and stallion plasma is less

than 100 mg./100 ml. Within a species the inositol level in the plasma varies considerably but the ratio of free to combined inositol appears, on the basis of the present limited series of estimations, to be approximately constant for each species. Thus in boar and human seminal plasma almost the entire inositol is present in the free state. The proportions of inositol in the free state in the plasma of bull and stallion semen are 55 and 85% respectively while for ram plasma, where the variations are greater, the figure is of the order of 30%. When two ejaculates were collected from a bull in a single day, the inositol concentration was markedly lower in the second ejaculate.

While the seminal vesicles are the main source of free inositol in boar semen (Mann, 1951, 1954*a*), the epididymal seminal plasma contributes significant quantities of both free and combined inositol; the inositol content of boar epididymal plasma is of the same order as the inositol content of seminal plasma in other species. Monkey seminal vesicle secretion is also comparatively rich in free inositol. On the other hand, the seminal vesicle secretion of the bull appears to contain rather less of both free and combined inositol than does the seminal plasma. Among the secretions so far examined the richest in combined inositol was a sample of stallion ampullar secretion (Table 3).

Table 2. *Free and total inositol in seminal plasma*

The two pairs 16, 17 and 18, 19 each represent first and second ejaculates respectively collected from the one animal on the same day. Sample 23 was from a bull which always provides semen containing separate sperm heads and sperm tails.

Species	Sample no.	Animal no.	Inositol (mg./100 ml.) Free	Total
Boar	2	I	382	—
	5	I	625	605
	7	II	607	725
Bull	12	I	27·2	48·0
	16	II	45·9	83·8
	17	II	28·1	44·9
	18	III	45·0	88·5
	19	III	38·6	61·4
	23	IV	24·6	52·1
Human	51	*	50·0	53·6
	52	*	57·3	62·8
Rabbit	34	I	21·0	27·7
Ram	1	*	9·7	37·3
	4	*	16·1	—
	6	*	14·6	39·0
	8	*	—	45·4
	9	*	7·4	41·5
Stallion	3	I	24·4	—
	10	I†	27·5	33·6
	11	I	19·0	24·0
	13	I	39·6	43·5
	14	I	47·3	51·2
	15	I	29·5	35·0

* Ten or more pooled ejaculates. † Three pooled ejaculates from the one animal.

Table 3. *Free and total inositol in male accessory secretions*

Species	Secretion or tissue	Sample no.	Inositol (mg./100 ml.) Free	Total
Boar	Epididymal seminal plasma	20	36·5	52·2
Bull	Seminal vesicle secretion	21	40·0	54·9
		22	18·7	25·5
Guinea pig	Seminal vesicle secretion	44	19·2	26·1
Monkey	Seminal vesicle secretion	26	145	141
Hedgehog	Prostate I and II*	32	17·5†	—
	Prostate III*	33	1·5†	—
Stallion	Ampullar secretion	25	20·4	93

* Whole gland. † mg./100 g. fresh weight.

The total inositol content of ram spermatozoa was found to be 180 mg./100 g. dry weight. Nevertheless, the lipids isolated from the same source by Lovern, Olley, Hartree & Mann (in preparation) were reported to be free from inositol lipids, although the analytical procedure used by these authors would have detected a quantity of inositol equivalent to as little as 10 mg./100 g. dry weight of spermatozoa. The initial stage in the separation of the lipids, exhaustive extraction with chloroform–methanol, removed less than half the total inositol in the dried spermatozoa. Of the inositol present in the resulting crude lipids virtually all was removed when they were subjected to the purification procedure of Folch, Ascoli, Lees, Meath & LeBaron (1951) followed by the cellulose-column treatment of Lea & Rhodes (1953) (unpublished results). Later experiments have shown that the residue remaining after chloroform–methanol extraction contains considerable quantities of bound inositol.

DISCUSSION

Apart from Mann's (1951, 1954*a*) published figures for the inositol content of vesicular secretion and semen of the boar the only previous information on inositol in semen was that human semen contains less than 100 mg./100 ml. (Nixon, 1952). The present results show that seminal inositol is of widespread occurrence. Although in most animals the level in the seminal plasma is 10–40 times lower than in boar seminal plasma, such levels are considerably higher than those characteristic of other body fluids, e.g. blood and cerebrospinal fluid (Table 4). Thus inositol at this higher concentration can be classified with citric acid (Scherstén, 1936) and fructose (Mann, 1946) as a characteristic component of semen.

On the basis of configurational relationships Fischer (1945) put forward the suggestion that inositol may be an intermediate in the interconversion of glucose and fructose. Since inositol is not readily degraded in animal tissues it could, according to this hypothesis, function as a non-metabolizable carbohydrate reserve. In this connexion can be cited certain parallelisms in the distribution of inositol and fructose: thus foetal blood and semen each contain unusually high concentrations of both substances (Table 4).

Inositol is well known as a growth factor for certain micro-organisms, while Woolley (1940, 1941*b*) has shown that it is an essential dietary factor for rats; the main deficiency symptom being alopecia. However, in later experiments Woolley (1942) found that the response of the rats to inositol-deficient diets was very irregular, a result which was traced to the ability of the intestinal flora to synthesize inositol. Availability of inositol from this source would explain why Needham (1924) failed to lower the inositol content of tissues of rats on an inositol-deficient diet. On the other hand, Eagle, Oyama, Levy & Freeman (1956) have demonstrated that inositol is a growth factor for certain normal and malignant cells in tissue culture. Of 19 cell lines (17 human, 2 mouse) 16 were unable to grow in absence of inositol and required concentrations of 10^{-5} to 10^{-6} M for optimum growth. Another established function for inositol is that of a lipotropic factor. Gavin & McHenry (1941) showed that inositol can prevent the formation of fatty livers in rats on high-cholesterol diets.

It is not possible at present to ascribe any metabolic function to inositol in semen or, in fact, in any other animal tissue. Feeding inositol to rats gives rise to no increase in liver glycogen but at the same time very little of the inositol is excreted in the urine. Posternak, Schopfer & Reymond (1955) have demonstrated the conversion by rats of deuterium-labelled inositol into 6-C labelled glucose, and Halliday & Anderson (1955) similarly established the formation of labelled inositol after feeding [1-^{14}C]glucose. However the rates of conversion were very low in both cases.

The materials used in the present work were, when not obtained from living animals, dissected out immediately after death. The importance of rapid

Table 4. *Mean values for fructose and free inositol in semen, cerebrospinal fluid, and maternal and foetal blood*

Apart from the results for the free inositol content of human and ram seminal plasma the results are quoted from the following papers: Bacon & Bell (1948); Campling & Nixon (1954); Cole & Hitchcock (1946); Eastham & Keay (1952); Mann (1954*b*); Nixon (1955); Wallenfels (1951).

	Fructose (mg./100 ml.)	Free inositol (mg./100 ml.)
Ram seminal plasma	300	15
Sheep blood, maternal	1	1·6
Sheep blood, foetal	100 (mid-pregnancy) 60 (at term)	20
Human seminal plasma	300	50
Human blood	2	0·6
Human cerebrospinal fluid	3·8	2·7

removal is emphasized by the work of Nixon (1955) who found dramatic rises in the inositol content of human cerebrospinal fluid within 12 hr. of death. These increases are ascribed to the hydrolysis of phospholipids present in the cytoplasm of the central nervous system. *Post mortem* autolytic rises of free inositol in muscle have also been reported (see Nixon, 1955).

The role of fructose as a nutrient for spermatozoa has been fully established (Mann & Lutwak-Mann, 1948), but attempts to demonstrate a similar role for inositol have been unsuccessful. Although fructose induces motility in epididymal spermatozoa, inositol does not. Furthermore, addition of inositol to boar epididymal semen or to washed sperm suspensions in Ringer–fructose solution does not bring about any change in respiration or fructolysis (Mann, 1954*a*).

The concentration of free inositol in boar vesicular secretion shows very little variation. Analysis of the fluids obtained from the vesicles of five boars gave an average figure of 2414 mg./100 ml. with a standard deviation of only 8 % (Mann, 1954*a*). On the other hand, the total volume of fluid obtained from a boar, as well as the combined weight of pairs of vesicles, varied more than tenfold. Since almost all the inositol in boar seminal plasma derives from the seminal vesicles, the inositol level in the plasma may prove to be a useful index of the contribution of the vesicular secretion to the ejaculated semen.

It has been pointed out by Mann (1954*a*) that boar vesicular secretion, unlike the other body fluids, is almost devoid of sodium chloride, and he suggests that the function of inositol here, and to a lesser degree in the seminal plasma, is to maintain the osmotic pressure. However, it is now established that although inositol is widely distributed in the accessory secretions which make up the seminal plasma, it is present at concentrations much lower than those characteristic of the boar (compare Table 2 of this paper with Table 4 of Mann, 1954*b*). It must therefore be assumed that, while the osmotic role of inositol in boar semen is important, this substance may well have some more specific function in semen.

SUMMARY

1. Free and total inositol have been assayed in seminal plasma, and in some accessory organs of the male reproductive tract, by a microbiological method which utilizes the yeast *Kloeckera brevis*.

2. Boar seminal plasma contains 600–700 mg. of inositol/100 ml., most of which is in the free state. Bull, human, rabbit, ram and stallion seminal plasma contain less than 100 mg./ml. In all species the inositol concentration is much greater than in blood and cerebrospinal fluid.

3. It is suggested that the inositol content of boar semen can be taken as an index of the secretory activity of the seminal vesicles.

4. A parallelism between the levels of fructose and inositol in body fluids supports the view that inositol may function as an intermediate in the interconversion of glucose and fructose, but at present there is no evidence for any metabolic role of inositol in semen.

I wish to express my thanks to Dr J. N. Hawthorne for gifts of inositol monophosphate and inositol phosphatide, and to Professor Th. Posternak for his gift of the homologous compounds listed in Table 1. I also wish to thank Dr T. Mann, F.R.S., for much helpful advice during the course of this investigation.

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Tissue Components of the Domestic Fowl

1. THE D-GLUCOSE CONTENT OF WHOLE BLOOD

By D. J. BELL
Agricultural Research Council Poultry Research Centre, Edinburgh 9

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Microanalyses of reducing sugars in avian bloods have been attempted many times with the general indication that the concentrations are markedly higher than those found in mammals. Most previous workers have used methods that are affected by *restreduktion* [non-carbohydrate blood components (cf. Herbert & Bourne, 1930)]. In a few instances 'fermentable sugar' has been estimated (Cassidy, Dworkin & Finney, 1926; Erlenbach, 1938; Fujita & Iwataki, 1931; Cavett, 1939). However, the addition of a washed yeast suspension to deproteinized blood, in which only few of the constituents have ever been identified, need not necessarily result in absorption of reducing sugars alone; other reducing components may be taken up by the yeast. Indeed, during fermentation, reducing substances can be formed [cf. Meyer (1913) and Merten (1938)].

The Folin & Wu (1919) method, as applied to the analysis of blood sugar in fowls, has been adversely criticized by Cassidy *et al.* (1926), Gulland & Peters (1930) and Fujita & Iwataki (1931). Ferricyanide methods, mainly that of Hagedorn & Jensen (1923), have also received adverse criticism by Gulland & Peters (1930), Erlenbach (1938) and Fujita & Iwataki (1931).

Few determinations of the blood sugar of fowls by titrimetric copper methods have been recorded. Strange, Dark & Ness (1955) have shown that both ferricyanide and copper reagents can be interfered with by amino acids as well as by creatine, uric acid, etc. and that Cd^{2+}, Ba^{2+} and Hg^{2+} will not satisfactorily remove these substances. These authors, likewise, found the copper reagents superior to ferricyanide.

Table 1 summarizes those papers known to the author where sufficient data have been given to allow comparison with the results of this paper. It is not claimed that this table is complete; because of indexing lacunae this field is difficult to search. The breeds of fowls, where known, are not stated in the table; there is no evidence so far of any influence of this factor on the blood sugar. It was therefore considered necessary, as a preliminary to future experimental work, that a search be made for a simple method which would determine the blood glucose in agreement with assays using D-glucose oxidase. The stabilized benzidine reagent of Jones & Pridham (1954) was found not to be affected by non-sugar components of three kinds of blood-'filtrates'; it gives results agreeing closely with blood-glucose determinations by means of D-glucose oxidase. The author is grateful to Professor A. St George Huggett, who drew his attention to the possibilities of the benzidine method (cf. Huggett, 1956).

METHODS

The birds used were Brown Leghorns taken either from pure lines or crosses from these lines, and were superficially healthy. No genetical influence on the blood-sugar levels was found. Unless fasted, the birds were allowed free access to the normal food used at this Centre.

This is a mash consisting of (in parts by weight) parings, 15·5; bran, 14·5; maize meal, 22·0; Sussex ground oats, 20·0; white fish meal, 9·5; dried unextracted brewer's yeast, 4·0; dried skimmed milk, 5·0; dried grass meal, 5·0; ground limestone, 1·0; common salt, 0·5; cod-liver oil (stabilized), 2·0; mineral mixture, 1·0. The last-named has the following composition (lb./approx. ton): exsiccated iron sulphate, 56; hydrated manganese sulphate, 124; limestone flour, 1399; sterilized feeding bone flour, 314; common salt, 336;