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# PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

*The* **283rd Meeting** *of the Biochemical Society was held at the British Postgraduate Medical School, Ducane Road, Hammersmith, London, W.* 12, *on Saturday,* 21 *January* 1950, *when the following papers were read:*

## COMMUNICATIONS

### Observations on the Chemistry of Hyaluronic Acid.

By M. A. G. KAYE and M. STACEY. (*Department of Chemistry, University of Birmingham*)

A modified technique has been devised for the isolation from human umbilical cord of hyaluronidin, a mucoprotein containing hyaluronic acid, and for isolating the latter, protein-free. D-Glucuronic acid, acetic acid and D-glucosamine, as components of hyaluronic acid, have been identified by isolation of derivatives and by the application of paper-strip chromatography.

Use of the methylation techniques, well-tried in polysaccharide chemistry, has failed so far in this case to give clear-cut results.

It appeared that in the relatively undegraded acid-polysaccharide macromolecule, only a relatively low content of hydroxyl groups were free to form methyl ether residues.

Degradation of the hyaluronic acid could be achieved under acid or alkali conditions to give a mixture of products some of which, on subsequent methylation, yielded methylated derivatives in the form of syrups, having relatively high methoxyl contents. Acid hydrolysis of these gave some products which were clearly monosaccharide derivatives but in the main the split products, particularly those fragments containing nitrogen, were oligosaccharide derivatives remarkably resistant to further acid hydrolysis. The only product which it has been possible to define was a dimethyl ether of methyl glucopyruronoside methyl ester, which, on further methylation, gave rise to fully methylated glucopyruronic acid (identified as the crystalline amide). It is possible to state that in the degraded material only very few, if any, of the glucuronic acid residues can be present as terminal groups and in these uronic acid units some of the carboxyl groups remain free and some are bound in either ester or amide formation or in some other way.

The methylated oligosaccharide units contain a relatively high proportion of nitrogen and appear to be closely related to chitin-like derivatives which form a remarkably stable 'core' in hyaluronic acid. From paper-strip chromatography it appeared also that nitrogen-containing units of a non-carbohydrate nature were present in hyaluronic acid. It is clear that techniques and methods new to carbohydrate chemistry will need to be developed in order to unravel the complex structure of hyaluronic acid.

It seems reasonably certain that the prevailing idea of hyaluronic acid as a polysaccharide acid containing alternating units of equal amounts of *N*-acetyl glucosamine and glucuronic acid is untenable. It is possible that the *N*-acetyl amino sugar units form a chitin-like 'core' to which is attached a complicated polyglucuronic acid structure and a prosthetic group.

Attempts are now being made to separate and identify labile residues by cautiously graded hydrolysis and to identify products obtained by the acetolysis technique.

### Observations on the Structure of the Specific Polysaccharide of *Azotobacter chroöcoccum*.

By G. J. LAWSON and M. STACEY. (*Department of Chemistry, University of Birmingham*)

The viscous acid polysaccharide synthesized by *Azotobacter chroöcoccum* (Cooper, Daker & Stacey, 1938) reacts in high dilution with Types III and VI pneumococcus antiserum (M. Heidelberger, private communication). This same behaviour is exhibited by the capsular polysaccharide of another nitrogen-fixing organism, namely *Rhizobium radicicolum* (clover strain) which was shown to be related structurally to the Type III pneumococcus polysaccharide inasmuch as it possessed a cellobiuronic acid type of unit (Schlüchterer & Stacey, 1945). The structure of the *Azotobacter* polysaccharide has now been examined by classical and chromatographic methods. Aqueous hydrolysis followed by paper chromatography revealed the presence of glucose, galactose and glucuronic acid residues only. Methylation with dimethyl sulphate and sodium hydroxide afforded an almost colourless homogeneous methyl

ether (OMe, 39 %) soluble in a mixture of chloroform and acetone giving highly viscous solutions. This ether was broken down in a stepwise manner by graded hydrolysis with methanolic HCl, yielding mixtures of methylhexosides and an oligosaccharide acid separated as a barium salt. The mixture of hexosides could not be satisfactorily separated by fractional distillation in a high vacuum owing to the formation of azeotropes, though it was possible to show the complete absence of fully methylated hexose 'end groups'.

The partially methylated hexoses in the azeotropes could not satisfactorily be converted into anilides, but the presence of D-glucose and D-galactose was confirmed by conversion to their crystalline tetramethyl derivatives.

A chromatographic examination (kindly carried out by Dr J. K. N. Jones) of the main part of hexoside fractions, on columns of cellulose (Hough, Jones & Wadman, 1949), showed them to consist of 2:3:6-trimethylglucose, together with smaller amounts of 2:4:6-trimethylglucose, 2:4:6-trimethylgalactose, with an as yet unidentified dimethylhexose(s). A similar examination of the glucuronic acid containing oligosaccharide showed it to be a trisaccharide type composed of 2:4:6-trimethylglucose, a dimethyl (possibly 2:3) glucose, and a partially methylated glucuronic acid.

The data, given with some reserve, indicated a possible repeating unit for the polysaccharide composed of three D-glucose units and one D-galactose molecule and one D-glucuronic acid unit. The D-galactose is linked through positions 1 and 3, while the glucose is linked partly through positions 1 and 4 and partly through positions 1 and 3. The polysaccharide clearly possesses a branched chain structure with the carboxyl groups of the uronic acid residues free to engage in salt formation, such a structure closely resembling that of the plant gums.

Similarities to the Type III pneumococcus polysaccharide structure (Reeves & Goebel, 1941) are shown in the possession of the glucose-glucuronic acid type of aldobiuronic acid residues and in the fact that both polysaccharides contain a significant proportion of 1:3-glycosidic linkage. The relationship to the *Rhizobium* polysaccharide lies in the aldobiuronic acid residues.

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## Microbiological Assay of Pure Vitamin $D_2$ and Vitamin $D_3$; Possible Function of Non-ionic and Ionic Lipids for the Bacterial Cell.

By E. Kodicek. (*Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council*)

The assay procedure has been based on previous findings of the bacteriostatic effect on *L. casei* of *cis*-forms of long-chain unsaturated fatty acids (Kodicek & Worden, 1944, 1945, 1946). The bacteriostasis could be reversed by several substances of which vitamins $D_2$ and $D_3$ were the most potent. The reversing effect of vitamin D was proportional to its concentration, within a range of 0·5–4 μg./ml. of medium. The technique required strict standardization of the inoculum and addition of an amount of linoleic acid (*c.* 20 μM) just sufficient to effect complete cessation of growth and acid production. Vitamin $D_3$ gave quantitatively the same response. The variation between experiments was considerable, nevertheless, it was no greater than the variation observed in the initial stages of other microbiological vitamin assays. Tests for specificity on seventy different substances showed that of the fat-soluble compounds, only cholesterol, vitamin E, lecithin, and to a lesser degree coprostanone, interfered (Table 1); work is in progress to ascertain whether the assay can be made specific for vitamin D. Promising results were obtained in preliminary trials with the non-saponifiable matter of some

Table 1. *Reversal effect by fat-soluble substances of linoleic acid inhibition*

| Active | Inactive |
|---|---|
| Vitamin $D_2$ | 7-Dehydrocholesterol |
| Vitamin $D_3$ | Lumisterol |
| | Dihydrocholesterol |
| Cholesterol | Cholestanone |
| | Ergosterol |
| | Zymosterol |
| | Sitosterol |
| | Coprostanone (±) |
| | Testosterone |
| | Desoxycorticosterone acetate |
| Lecithin | Lecithin, saponified |
| α-Tocopherol | α-Tocopherol, saponified(±) |
| | Vitamin A alcohol |
| | Vitamin A acetate |
| [$Ca^{++}$] | |

natural products containing vitamin D; fish liver oils, however, contained a potent inhibitor which interfered with the assay.

Apart from the practical application, the theoretical aspect is of interest. The bacteriostatic effect of linoleic acid can be explained primarily on a physico-chemical basis (barrier theory; Kodicek, 1948, 1949) which does not exclude the possibility that unsaturated fatty acids have also a metabolic function. By the adsorption and penetration of linoleic acid into the lipoprotein patches of the cell wall of the bacteria, the surface pressure is increased and may distort spatially and functionally the protein lattice. Such substances as vitamin D reverse this effect. Small amounts of vitamin D (*c.* 10–20 $\mu$M) in absence of linoleic acid were toxic, and so were high amounts of the vitamin even in the presence of linoleic acid. The growth-promoting properties of vitamin D and of calcium salts and the toxic action of the former are reminiscent of similar findings in higher organisms.

The author wishes to thank Prof. Friedemann and Mr A. L. Bacharach for their precious gift of various sterols and Mr D. R. Ashby for his valuable technical assistance.

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**The Metabolism of Halogenobenzenes. Isolation of a Dihydrodihydroxychlorobenzene and other Metabolites from Chlorobenzene Urine.** By B. Spencer and R. T. Williams. (*Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2*)

In the past, studies on the metabolism of the halogenobenzenes have been mainly concerned with their ability to form mercapturic acids. These acids, however, only account for about 20–25% of the halogenobenzenes fed. The present work shows that the main bulk of these compounds are oxidized in the rabbit and twice as much of the halogenobenzenes are excreted as O-conjugates as in the form of S-conjugates. The chlorophenols form no mercapturic acids and give only O-conjugates (see Table 1).

Chlorobenzene urine has been examined in detail and some of the metabolites isolated and identified as follows:

(*a*) *Diol of chlorobenzene.* Chlorobenzene urine was adjusted to pH 7·5 and continuously extracted with ether for 16 hr. Acidic constituents were removed from the extracts with alkali; from the dried ether extract *dihydroxydihydrochlorobenzene* (m.p. 129–130° and $[\alpha]_D - 413°$ in water) was isolated. (Found: C, 49·1; H, 4·7; Cl, 24·1%; $C_6H_7O_2Cl$ requires C, 49·1; H, 4·8; Cl, 24·2%.) The yield was minute, being 20·5 mg. from 90 g. of chlorobenzene fed. It was shown spectroscopically to be a $\Delta^{1:3}$-hexadiene compound which was completely dehydrated by 0·1 N-HCl in 35 min. at 98° to *p*-chlorophenol. This suggests that it is *l*-3:4-dihydroxy-3:4-dihydrochlorobenzene.

(*b*) p-*Chlorophenol and* 4-*chlorocatechol.* These were isolated in small yields by direct ether extraction of the urine at pH 5. The two phenols were separated by lead acetate precipitation and identified as *p*-toluene-sulphonates. The same phenols were isolated in good yields after mild acid hydrolysis of the urine; the yields of 4-chlorocatechol and *p*-chlorophenol were in the ratio of 20 to 1.

Table 1

| | Percentage of dose excreted as | | |
|---|---|---|---|
| | O-conjugates† | | S-conjugates† |
| Compound* | Ethereal sulphates | Glucuronides | Mercapturic acid |
| Chlorobenzene | 27 | 25 | 20 |
| Bromobenzene | 37 | 40 | 21 |
| Iodobenzene | 30 | 31 | 23 |
| *o*-Chlorophenol | 19 | 82 | 0 |
| *m*-Chlorophenol | 21 | 68 | 0 |
| *p*-Chlorophenol | 13 | 88 | 0 |

* Doses in molecular equivalents of 150 mg. chlorobenzene/kg.

† Assuming that monoconjugates are formed.

(*c*) p-*Chlorophenylglucuronide.* This substance wa obtained in small yield from the glucuronide gum and characterized as its *amide* (m.p. 245–246° decomp.; $[\alpha]_D - 85·6°$ in methanol). This amide was also prepared biosynthetically by feeding *p*-chlorophenol to rabbits.

(*d*) *Glucuronide of* 4-*chlorocatechol.* This substance was isolated in large yield after gentle acid hydrolysis of the urine or of the glucuronide gum. It was characterized as 4-chloro-2-methoxyphenylglucuronidamide and was identical with the glucuronide

obtained from the urine of rabbits fed with 4-chlorocatechol (Dodgson & Williams, 1949). Our experiments suggest that this compound is an artefact but the nature of its precursor has not yet been established.

(*e*) p-*Chlorophenylmercapturic acid*. This wasi solated during prolonged ether extraction of chlorobenzene urine and had m.p. 153° and $[\alpha]_D -20°$ in ethanol.

The above results were discussed.

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**The Interactions of Purified Serum Albumins with Calcium.** By N. H. Martin and D. J. Perkins. (*Department of Chemical Pathology, St George's Hospital, London, S.W.* 1)

MacLean & Hastings (1935) first attempted a quantitative study of the relationship of calcium to horse and bovine albumins prepared by fractionation of the original sera with ammonium sulphate and further purification by electrodialysis. Further studies were carried out by Weir & Hastings (1936) and by Drinker, Green & Hastings (1939). In the last ten years there have been considerable advances in the techniques of protein fractionation and purification. It seemed useful, therefore, to reinvestigate the problem with the products now available.

In the present investigation albumins of varying purities from three different species, horse, ox and human, were examined. Though the purity of each specimen was assessed by ultra-centrifugal and electrophoretic analysis, it was appreciated that the processes by which separation had been achieved might result in modifications of the intimate structure of the albumin molecule not appreciable by either of these physico-chemical techniques. For this reason albumins separated by a variety of techniques from each species were studied. In all five different samples of human albumin, one of horse, and three of bovine, were examined.

Preliminary investigations showed that, under the standard conditions chosen, equilibrium was achieved in four days. Seven days were allowed in each of the groups of experiments reported. The pH of the system was measured when equilibrium had been achieved, the Donnan effect was determined experimentally and a correction applied, and, in every case, interactions were studied over a range of protein concentrations.

Table 1

| Albumin | Separation | Approximate purity electrophoretic analysis (%) | Ca mg./Protein N g. (mean of readings) | Range of proteins conc. studied (g./100 ml.) | pH range studied |
|---|---|---|---|---|---|
| 1. Horse | Crystallized. Kekwick. Carbohydrate-poor fraction* | 100 | 11·8 | 0·7–2·8 | 6·3–6·5 |
| 2. Ox (*a*) | Pillemer alcohol fractionation | 94–97 | 11·2 | 1·4 | 6·8 |
| 3. Ox (*b*) | Armour preparation | 90–95 | 4·1 | 6·0 | 6·9 |
| 4. Ox (*c*) | Crystallized from alcohol fractionation† | 100 | 2·2 | 2·8–5·6 | 6·1–6·3 |
| 5. Human (*a*) | Pillemer alcohol fractionation | 94–97 | 4·7 | 1·4–3·0 | 6·6–6·7 |
| 6. Human (*b*) | Ether fractionation of Kekwick and MacKay* | 85 | 4·8 | 2·0–4·0 | 6·6–6·7 |
| 7. Human (*c*) | 25% albumin (American Red Cross) for therapy | 95–97 | Nil | 6·0 | 5·8–8·4 |
| 8. Human (*d*) | Five times recrystallized alcohol fractionation† | 100 | 4·7 | 2·5–5·0 | 6·7–6·85 |
| 9. Human (*e*) | Decanol recrystallization from alcohol fractionation† | 100 | 1·1 | 1·8–5·0 | 6·2–6·4 |

* We are indebted to Dr Kekwick for these specimens.
† We are indebted to the Department of Physical Chemistry, Harvard, for these specimens.

It will be seen that the combining power of horse and ox albumins are of the same order, and are substantially greater than that of the human albumins purified and examined under comparable conditions.

It demonstrates further that repeated crystallization may alter the capacity of the albumin to bind calcium, either by modification of the molecule or by removal of a specific fraction responsible for calcium binding, and that techniques of purification vary in their effect as measured by calcium binding.

Because stabilizers might have been used in the preparation of albumin No. 7 (Boyer, Lum, Ballou, Luck & Rice, 1946), the effect of the presence of acetyl tryptophane on the binding capacity of other albumins was investigated but no interference could be demonstrated.

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**A Biochemical Description of Locust Coloration.** By T. W. Goodwin and S. Srisukh. (*Department of Biochemistry, The University, Liverpool* 3)

Immature adult (male and female) Desert Locusts (*Schistocerca gregaria*) are predominantly pink, whilst mature males are yellow and mature females are brown. The two major pigments in these locusts are $\beta$-carotene (Goodwin & Srisukh, 1949; Goodwin, 1949) and acridioxanthin; the latter being a redox pigment of unknown constitution first mentioned by Chauvin (1941). In the immature and mature adults of both sexes there is very little variation in the amounts of these pigments present, so qualitative differences must be sought to explain the colour changes. It has been found that the pink of the immature insects is due to the presence of free reduced acridioxanthin, the brown of the mature females to reduced acridioxanthin attached to a tissue constituent, in all probability a protein, and the yellow of mature males to $\beta$-carotene which is revealed, in the body segments at least, partly by the withdrawal of the acriodioxanthin to the edges of the segments and probably also partly by the translocation of the $\beta$-carotene from the subcutaneous tissues into the hypodermis.

When the complex colour patterns of the integument of the pink and brown insects are considered it is found that the patterns are produced merely by the differential accumulation of acridioxanthin over the surface of the integument; even the very dark narrow markings of the legs are due to the same pigment. 'Melanin' is apparently confined to the wings, mandibles and leg joints. Similar considerations can be applied to the Migratory Locust (*Locusta migratoria migratorioides*).

The brown patches found on some solitary locusts are also produced by acridioxanthin, but the frequently occurring green patches contain a complex (very similar to Junge's (1941) 'insectoverdin') composed of orange-yellow astaxanthin and $\beta$-carotene chromoproteins together with a blue chromoprotein with probably glaucobilin as its prosthetic group, and a yellow water-soluble substance.

Coloured plates were produced to illustrate these findings.

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**Utilization of a Casein Supplement Administered to Ewes by Ruminal and Duodenal Fistulae.** By D. P. Cuthbertson and Margaret I. Chalmers. (*Rowett Research Institute, Bucksburn, Aberdeenshire*)

When casein is fed or administered into the rumen of a sheep there is an immediate rise in the ammonia concentration of the rumen which reaches a peak in 2 hr. and then steadily declines (McDonald, 1948*a*). A rapid rise occurs in the ammonia concentration of the venous blood draining the rumen (McDonald, 1948*b*) after the administration of casein into the rumen (McDonald, 1948*a*).

It was found that pregnant ewes on different levels of nutrition excrete 60–70 % of the nitrogen supplied from a casein supplement of 50 g. per day by mouth. Nitrogen balance experiments on sheep receiving the casein supplement dripped through fistulae into the duodenum and rumen, show that the percentage retention of the casein-N when given by duodenal fistula is greater than that from casein dripped into

Table 1. *Nitrogen retained from a casein supplement of* 50 *g. per day*

| Sheep | | | Basal N intake (g./day) | N intake + casein (g./day) | % N retained by (days of period in brackets) | |
|---|---|---|---|---|---|---|
| | | | | | Duodenum | Rumen |
| N | Low-plane* | Pregnant | 6·06 | 12·21 | 49 (12) | 18 (7) |
| O | Low-plane | Pregnant | 6·06 | 12·21 | 64 (7) | 41 (12) |
| P | Low-plane | Pregnant | 6·06 | 12·21 | 50 (19) | — |
| Q | Low-plane | Pregnant | 6·06 | 12·21 | — | 34 (18) |
| R | Low-plane† | Non-pregnant | 9·20 | 15·66 | 18 (9) | Out>in§ (9) |
| S | Low-plane | Non-pregnant | 9·20 | 15·66 | 24 (9) | 20 (9) |
| N | Well-fed‡ | Non-pregnant | 24·90 | 31·40 | Out>in (15) | 6 (15)<br>8 (15) |
| O | Well-fed | Non-pregnant | 24·90 | 31·40 | 17 (15)<br>4 (15)<br>9 (15) | — |
| P | Well-fed | Non-pregnant | 24·90 | 31·40 | 20 (15)<br>11 (15) | Out>in (15) |
| Q | Well-fed | Non-pregnant | 24·90 | 31·40 | — | 13 (15)<br>Out>in (15)<br>1 (15) |

* Diet = meals 62½ g.; hay 250 g.; swedes 750 g. per day.
† Diet = meals 150 g.; hay 400 g. per day.
‡ Diet = meals 550 g.; hay 900 g. per day.
§ More N excreted than ingested from the casein supplement.

the rumen. The extent to which the nitrogen was retained also depended on the state of nutrition of the animal. Data are shown in Table 1.

It would appear that the rate of deamination of the casein in the rumen was in excess of the capacity to synthesise bacterial protein and therefore the ammonia formed was absorbed into the blood stream, converted to urea and excreted in the urine. Thus the low-plane pregnant ewes failed to use the casein supplement to meet their requirement for protein. In the well-fed animals the food consumed was sufficient to cover any loss due to deamination of protein in the rumen.

We wish to thank Dr A. T. Phillipson for carrying out the fistulae operations on the animals.

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**Experiments on the Mechanism of the Formation of Methylthiol from *S*-methyl-L-Cysteine in Cultures of *Scopulariopsis brevicaulis*.**
By F. Challenger and A. G. Lowther. (*Department of Organic Chemistry, The University, Leeds*)

One of us and Charlton showed (1947) that in bread cultures of *Scopulariopsis brevicaulis* (Strain, Washington 2; N. Coll. T.C. No. 580) DL-methionine gives methylthiol and, by further methylation, dimethyl sulphide in (we find) a total yield of 15 %. Similarly, *S*-alkyl-L-cysteines (alkyl = Me, Et and *n*-Pr) give alkylthiol and methylalkyl sulphide, in (we find) a total yield of 80 %, with *S*-methyl-L-cysteine. Glucose-Czapek-Dox (G.-C.-D.) medium, adjusted to pH 7, has been used for a study of the mechanism of the *S*-methyl-cysteine fission by the same strain of *S. brevicaulis*. This may be (*a*) reductive, (*b*) hydrolytic or (*c*) may involve the elimination of $CH_3S$- and H- from adjoining carbon atoms, giving different products in each case. Alternatively, the α-keto acid $CH_3SCH_2COCOOH$ may be a primary product and then undergo (*a*) or (*b*).

The mould was grown in several flasks of the adjusted G.-C.-D. medium (200 ml.) containing 0·25 % of *S*-methyl-L-cysteine. A flask was removed every 7–10 days, the contents filtered, concentrated, sterilized (autoclave or Seitz filter gave the same results) desalted and the medium examined by two-dimensional paper chromatography (phenol, collidine). In control experiments without *S*-methyl-cysteine, the cultures were similarly examined.

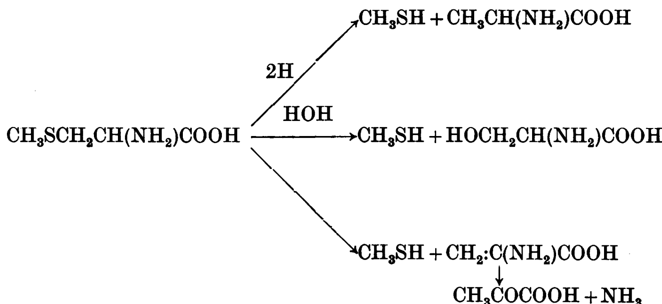
The first test-culture showed glycine, valine and leucine in fairly low concentration, aspartic acid and alanine in fairly large concentration and much *S*-methylcysteine. Later tests gave similar results, but the amount of amino-acids decreased and after 6 weeks aspartic acid only was found. No serine was ever detected. The control experiments revealed only a fairly high concentration of aspartic acid.

The electrolytic desalting apparatus was shown to produce no alanine from *S*-methyl-L-cysteine.

The identification of alanine was confirmed by addition of authentic alanine to the solution to be examined, when the concentration, as shown by the intensity of the ninhydrin colour reaction, was increased and the two spots were exactly superimposed.

four days. The pyruvic acid was recovered as its 2:4-dinitrophenylhydrazone. It appears reasonable to conclude that the disappearance of the alanine is due to its conversion to protein.

These preliminary results have no quantitative significance, but the absence of serine and of pyruvic acid and the relative intensity of the alanine 'spot'




The disappearance of the alanine (also observed on addition of alanine to G.-C.-D. cultures of the mould) may be due to utilization in protein formation or to oxidative deamination to pyruvic acid. This was not detected by alkali titration or addition of 2:4-dinitrophenylhydrazine. Pyruvic acid added to cultures on G.-C.-.D medium did not disappear (tests as before) during 21 days in agreement with the results of Barnard (1947), who found no fall in alkali titre and no formation of acetaldehyde during

suggest a reductive fission of methylcysteine. It is hoped, following a suggestion by Dr C. E. Dent, to examine the behaviour of *S*-methyl-DL-cysteine with the mould. As D-alanine would presumably not be utilized in protein synthesis it—or possibly pyruvic acid arising by deamination—might be expected to accumulate.

We gratefully acknowledge the help of Dr Dent who allowed one of us to work in his laboratory, and also the interest of Dr W. S. Reith.

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**The Seasonal and Species Distribution of Oestrogen in British Pasture Plants.** By S. P. LEGG (*The National Institute for Research in Dairying, Shinfield*), D. H. CURNOW and S. A. SIMPSON (*The Courtauld Institute of Biochemistry, Middlesex Hospital, London, W.* 1)

A review of the literature (Löve & Löve, 1945) points out that the occurrence of oestrogens in plants was first observed by Loewe & Spohr (1926), and that the animal sex hormones have since been demonstrated in plant material ranging from the bacteria to the flowering plants. The role of these substances in plants has not been fully explained, but that they may affect the reproductive system of the grazing animal and cause infertility has been demonstrated by Bennetts and his collaborators (Bennetts, 1944; Bennetts, Underwood & Shier, 1946). These workers devised a method of extraction and assay which made quantitative tests on plant material possible (Robinson, 1949). A preliminary examination which demonstrated the presence of oestrogens in English pasture has already been reported (Bartlett, Folley, Rowland, Curnow & Simpson, 1948). A more detailed investigation is in progress, and results obtained during 1949 are now reported. The method of extraction and assay has been essentially that of Robinson. The chemical nature of the oestrogen and the state in which it occurs in the plant are not yet known, and there is some doubt as to the efficiency of the extraction. Table 1 shows that considerable oestrogenic activity has been found both in grasses and clover. Lucerne (*Medicago sativa* L.), White Clover (*Trifolium repens* L.) and Timothy Grass (*Phleum pratense* L.), however, showed little activity. In each of the potent species, activity has been found in all parts of the plant tested, i.e. leaf, petiole, stem and inflorescence. There is considerable seasonal variation and the various plant organs do not show maximal concentrations simultaneously. It has also been found that when chloroplasts are precipitated from the juice obtained by crushing plant material, the bulk of the oestrogen occurs in the precipitate.

The results indicate that oestrogens occur in British pasture plants in concentrations which vary with the species. The early occurrence in the leaf and

Table 1. *Occurrence of oestrogens in Ryegrass, Cocksfoot and Broad Red Clover during the* 1949 *growing season*

(Oestrogenic activity is expressed as μg. oestradiol per 100 g. plant dry matter.)

| | | Date of sample during 1949 | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Feb. | | Mar. | | Apr. | | May | | June | | |
| Plant | Description of material | 1st half | 2nd half | 1st half | 2nd half | 1st half | 2nd half | 1st half | 2nd half | 1st half | 2nd half | Other samples |
| Perennial Ryegrass S 23 (*Lolium perenne* L.) | Leaf | — | — | — | — | 4·5 | — | 4·5 | 0·2 | 0·2 | — | 0·3* |
| | Stem | — | — | — | — | 3·2 | — | 5·0 | 0·8 | Nil | — | |
| H.I. Ryegrass | Leaf | — | 0·6 | — | 10·0 | 1·1 | — | — | — | — | 0·4 | |
| | Stem | — | — | — | 2·3 | 0·7 | — | — | — | — | Nil | |
| Cocksfoot S 37 (*Dactylis glomerata* L.) | Leaf | — | — | — | 1·5 | 2·4 | — | 1·4 | 0·4 | Nil | 0·3 | Nil† |
| | Stem | — | — | — | Nil | Nil | — | 0·1 | 2·3 | Nil | — | |
| | Inflorescence | — | — | — | — | — | — | — | — | 1·3 | — | |
| Red Clover (*Trifolium pratense*) | Leaf | — | 2·2 | 4·0 | — | — | — | — | — | 3·2 | 2·4 | 2·7‡ |
| | Stem | — | — | 1·9 | — | — | — | — | — | 0·9 | 0·9 | |

* 3 Oct. 1949. † 13 July and 3 Oct. 1949. ‡ 26 Nov. 1948.

the high concentration in chloroplasts suggest that this is the site of production. Various workers have shown that application of oestrogens stimulates vegetative growth (Bonner & Axtman, 1937; Orth, 1934; Zollikofer, 1936; see also Löve & Löve, 1945), but the findings listed above suggest that the high oestrogen concentration is associated with reproductive growth. Spring growth of grass, which is associated with high oestrogen concentration, precedes flowering but when autumn growth takes place the plant is not in a reproductive phase. Broad Red Clover, on the other hand, which has repeated high concentrations of oestrogen, will produce flowers throughout the summer and autumn.

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## The Amino-Acid Pattern in Human Foetal and Maternal Plasma at Delivery.

By H. R. Crumpler, C. E. Dent and O. Lindan. (*Medical Unit, University College Hospital, London, W.C.* 1)

Foetal and maternal blood collected from nine cases at the moment of birth has been analysed by the ninhydrin/$CO_2$ method (Hamilton & Van Slyke, 1943) for total α-amino-nitrogen ($NH_2$-N) and by paper chromatography (Consden, Gordon & Martin, 1944) for its content of individual amino-acids. The foetal plasma contained a higher total concentration of $NH_2$-N than the maternal.

The increase involved all the amino-acids commonly found in plasma. These results support the view that the placenta, by concentrating the amino-acid content of foetal blood, assists the foetus to synthesize proteins (Christensen & Streicher, 1948).

The lower foetal/maternal ratios (1·4 or less) for the plasma $NH_2$-N occurred in the four cases in which the mother had definite toxaemia of pregnancy. It is suggested that this may indicate that some impairment of placental function occurs in toxaemia.

| | Foetal $NH_2$-N (mg./100 ml.) | Maternal $NH_2$-N (mg./100 ml.) | Ratio $\frac{\text{Foetal } NH_2\text{-N}}{\text{Maternal } NH_2\text{-N}}$ |
|---|---|---|---|
| Mean | 4·62 | 2·99 | 1·60 |
| Range | 2·94–6·70 | 2·23–3·70 | 1·03–3·00 |

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**The Relationship of Acridanes to the Metabolism of Mepacrine in Man.** By A. L. Tárnoky. (*Postgraduate Medical School, London, W.* 12 *and the Group Laboratory, Mile End Hospital, London, E.* 1)

The urine of subjects on mepacrine therapy contains substances which on spectrographic evidence may be diphenylamine or acridane derivatives (King, Gilchrist & Tárnoky, 1946). While the former are more stable, the latter should be formed more readily, and the deciding factor is the stability of acridanes in solution.

The rates of decomposition of acridane and 2-chloro-7-methoxyacridane at room temperature have been studied by an iodometric method. The results show the instability of these compounds which is most marked in acid solution. Thus when 0·01 M solutions in 90% ethanolic 0·1 N-HCl are prepared, 71% of acridane and 70% of 2-chloro-7-methoxyacridane are lost in the first 24 hr. The instability of these compounds also interferes with the determination of their absorption spectra and acid solubilities. There are signs that the reaction is not always a simple oxidation to the corresponding acridine.

It is concluded that simple (5:10-unsubstituted) acridanes may be ruled out as the metabolic end-products of mepacrine as suggested by the spectral curves.

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**The Nature of 'Inactive Haemoglobin'.** By I. D. P. Wootton. (*Postgraduate Medical School, London, W.* 12)

**Studies on 5-Nucleotidase and its Distribution in Human Tissues.** By J. L. Reis. (Introduced by E. J. King.) (*Postgraduate Medical School, London, W.* 12)

The 5-nucleotidase is a phosphatase with a specific action: it acts only on the nucleotides with a phosphate group attached to the fifth carbon of the pentose, that is on the adenylic acid (adenosine-5-monophosphoric acid) and on the inosinic acid (inosine-5-monophosphoric acid). Its high degree of specificity was demonstrated in previous papers (Reis, 1937, 1939). It does not act on adenosinetriphosphoric acid, ribose-5-phosphoric acid, adenosine-3-monophosphoric acid or on any other phosphoric ester. The activity of the 5-nucleotidase is estimated by comparing the rates of hydrolysis of adenylic acid and phenylphosphoric acid. The latter is, as found by King & Delory (1939), a much more suitable substrate for the phosphatase than the $\beta$-glycerophosphoric acid, used previously for that purpose. Naturally the hydrolysis of the adenylic acid represents the sum of the 5-nucleotidase and phosphatase actions.

For comparison of 5-nucleotidase and phosphatase rabbits' lungs were used, as both enzymes are present in this tissue. As no method of separation of the 5-nucleotidase from the phosphatase could be found, for studying the 5-nucleotidase alone, a tissue must be chosen where that enzyme is abundant and the phosphatase present in traces only. Such conditions were found in man in the walls of the aorta and in the thyroid gland. The pH optimum of the 5-nucleotidase for those tissues was found between 7·5 and 8·0. An activation by the Mg ion could be demonstrated. The optimal substrate concentration is much lower for the 5-nucleotidase than for the phosphatase. Whereas according to Neumann (1949) the lowest optimal substrate concentration for the alkaline phosphatase is M/300, for the 5-nucleotidase the lowest value in some extracts is M/2400, but even at M/4800 the rate of hydrolysis is not appreciably smaller.

In human tissue the 5-nucleotidase is widely distributed. In bones, the typical site of the alkaline phosphatase, though the alkaline phosphatase at its optimal pH is several times more active than the 5-nucleotidase, at physiological pH's this condition is reversed, the adenylic acid being five times more

Table 1. *5-Nucleotidase and phosphatase activity of human tissues*

($\mu$g. of phosphorus hydrolysed as phosphate, by an amount of extract corresponding to 20 mg. of tissue in 30 min. at 38°, at pH 7·5, substrate concentration M/1200.)

| Tissue | Phenylphosphoric acid | Adenylic acid | Ratio: adenylic acid hydrolysis to phenylphosphoric acid hydrolysis |
|---|---|---|---|
| Ossifying cartilage | 6 | 29 | 5 |
| Kidney | 7 | 17 | 2·5 |
| Liver | 1 | 9 | 9 |
| Spleen | 3 | 8 | 3 |
| Lungs | 3 | 18 | 6 |
| Walls of aorta | 0·5 | 34 | 68 |
| Brain | 0·5 | 15 | 30 |
| Choroid plexus | 3 | 24 | 8 |
| Muscle (skeletal) | 0·5 | 2 | 4 |
| Pancreas | 1 | 18 | 18 |
| Thyroid | 1 | 52 | 52 |
| Testicle | 1 | 38 | 38 |

easily hydrolysed than the phenylphosphoric acid. The 5-nucleotidase together with the phosphatase can be found also in lungs, kidney, brain and intestine. In nerves, walls of aorta, thyroid gland and in the testicles a 5-nucleotidase nearly free from phosphatase can be found (Table 1).

Taking into consideration that the phosphatase substrate in Robison's bone calcification theory (1932) is formed mainly by the adenylic acid (or by ATP) the importance of the 5-nucleotidase is obvious. It explains why calcification may proceed at physiological pH's in spite of the relative inactivity of the alkaline phosphatase in these conditions. Also in other tissues where calcification processes often take place (lungs, aorta, choroid) the presence of 5-nucleotidase can explain the chemical mechanism of the calcification on similar lines to that of the phosphatase in Robison's calcification theory.

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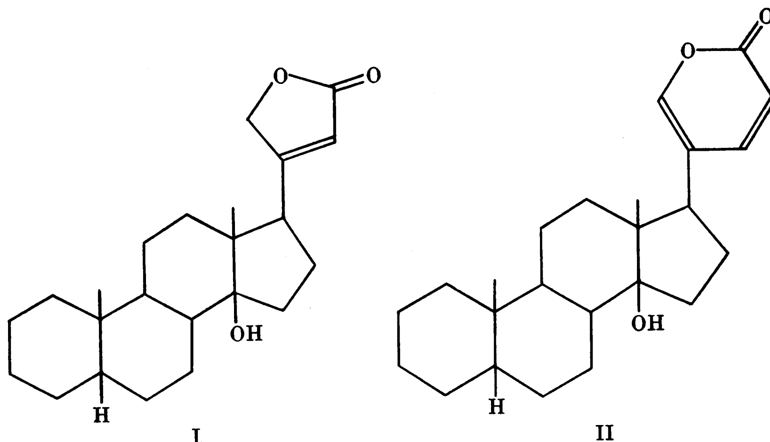
**The Molecular Rotations of Cardiac Aglycones and Toad Poisons.** By W. Klyne. (*Postgraduate Medical School, London, W.* 12)

The application of the method of molecular rotation differences to steroids containing many substituents is complicated by the fact that adjacent substituents may interfere with one another's rotation contributions ('vicinal action'; cf. Barton & Cox, 1948).

The rotation data for the cardiac aglycones and toad poisons (structures of Types I and II respectively) have now been analysed. The results show that, in general, substituents in ring D and in the side-chain do not interfere with the rotation contributions ($\Delta$ values) of substituents in the A and



B rings (at C-3, C-5 and C-19). Some of these Δ values have been given by Barton & Klyne (1948); others are given in Table 1A. Conversely, substituents at C-3, C-5 and C-19 do not in general interfere with the Δ values of substituents in the D ring and side-chain (see Table 1B).

Since the Δ values given in Table 1 are approximately constant, they may be used in elucidating the structures of aglycones and toad poisons substituted only in rings A, B and D.

The few data available for compounds substituted at C-11 and C-12 show clearly that Δ values at these positions for 14-*n* compounds differ considerably from those for 14-*iso* compounds. Δ values for some C-ring substituents in 14-*iso* compounds may be independent of the nature of the side-chain.

Table 1. *Molecular rotation differences*

(Δ value = molecular rotation difference = $[M]_D$ of *X minus* $[M]_D$ of *Y*. Experimental error for Δ values is about ±10°. All data are for 5-*n* compounds, and are taken largely from recent papers by Reichstein, Meyer and their co-workers.)

| Structure *X* | Structure *Y* | Δ value for *X*–*Y*: No. of examples | Range | Mean |
|---|---|---|---|---|
| | A. Structural changes in A and B rings | | | |
| $3(\beta):5(\beta)\text{-}(OH)_2$ | $3(\beta)$-OH | 4 | +41 to +51 | +46 |
| $3(\beta)$-OAc, $5(\beta)$-OH | $3(\beta)$-OAc | 5 | +107 to +128 | +120 |
| $3(\beta)$-OAc, $5(\beta)$-OH | $3(\beta):5(\beta)\text{-}(OH)_2$ | 5 | +78 to +93 | +87 |
| $3(\beta):5(\beta)\text{-}(OH)_2$, 19-CHO | $3(\beta):5(\beta)\text{-}(OH)_2$ | 2 | +54 to +58 | +56 |
| $3(\beta)$-OAc, $5(\beta)$-OH, 19-CHO | $3(\beta):5(\beta)\text{-}(OH)_2$, 19-CHO | 2 | +80, +80 | +80 |
| | B. Structural changes in D ring and side-chain | | | |
| $14(\beta)$-OH-14-*iso* aetio ester | 14-*n*-aetio ester | 3 | −45 to −66 | −53 |
| Structure I | $14(\beta)$-OH-14-*iso* aetio ester | 4 | −33 to −56 | −44 |
| Structure II | Structure I | 7 | −96 to −113 | −103 |

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**Preparation, Titration Curves and Enzyme Hydrolysis of Carboxyphenylphosphates.** By P. G. WALKER and E. J. KING. (*Postgraduate Medical School, London, W.* 12)

**Some Observations on the Splanchnic Blood Flow in Man, Using the Hepatic Vein Catheterization Technique.** By A. G. BEARN, BARBARA H. BILLING, J. C. S. PATERSON and SHEILA SHERLOCK. (Introduced by E. J. KING). (*Postgraduate Medical School, London, W.* 12)

## DEMONSTRATIONS

**An Apparatus for the Continuous Extraction of Reducing Steroids from Urine.** By J. P. NEWHOUSE and W. KLYNE. (*Postgraduate Medical School, London, W.* 12)

The acidified urine is extracted with chloroform in a liquid-liquid extractor, based on that of Robinson & Warren (1948) which was devised to avoid the persistent emulsions produced by hand-shaking in separating funnels. The extractor has been rendered automatic and self-adjusting by the use of a filter-pump, following the principle of the soil percolator of Lees (1948).

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**King-Armstrong Phosphatase Estimation by the Determination of Liberated Phosphate.** By
E. J. King, M. A. M. Abul-Fadl and P. G. Walker. (*Postgraduate Medical School, London, W.* 12)

The phenylphosphate method of phosphatase estimation (King & Armstrong, 1934) utilizes the determination of enzymically hydrolysed phenol as a measure of the phosphatase activity of blood plasma and tissue extracts. For some purposes it is preferable to determine the liberated phosphate instead of the phenol, e.g. in the study of rickets in children where a knowledge of both the phosphatase and the inorganic phosphate of the plasma is useful, and in certain studies of the kinetics of phosphatase action. Since, in the hydrolysis of phenylphosphate, equimolecular amounts of phenol and phosphate are liberated, therefore, approximately 3 mg. of phenol (mol. wt. 94) are hydrolysed for each 1 mg. of inorganic phosphorus (at. wt. 31). The King-Armstrong unit of phosphatase is defined as the amount of enzyme which liberates 1 mg. of phenol in 30 min. at pH 9 and 37°, or in 15 min. at pH 10 and 37° (King, 1947). Using the same conditions of hydrolysis it is consequently necessary to multiply the number of mg. of liberated P by three, in order to obtain the same unitage of phosphatase. The following procedure gives close agreement with phosphatase determinations made by the estimation of phenol according to the phenylphosphate method.

*Test:* 3 ml. of 0·1 M-sodium carbonate-bicarbonate buffer pH 10 ($6Na_2CO_3$:$4NaHCO_3$) *plus* 3 ml. of 0·01 M-disodium phenylphosphate, warmed at 37° for 3 min.; 0·3 ml. plasma added, mixed and kept at 37° for 15 min.; removed from 37° bath and 1·2 ml. trichloroacetic acid (20 %) added to stop hydrolysis and precipitate proteins.

*Control:* 6 ml. water and 0·3 ml. plasma *plus* 1·2 ml. trichloroacetic acid.

*Blank:* 3 ml. buffer, 3 ml. phenylphosphate, 0·3 ml. water and 1·2 ml. trichloroacetic acid.

The mixtures are centrifuged or filtered, and 5 ml. filtrate (≡0·2 ml. plasma) treated with 0·8 ml. of 5 % ammonium molybdate in 15 % (v/v) $H_2SO_4$ and 0·2 ml. aminonaphtholsulphonic acid-reducing agent (0·2 of the 1:2:4-acid, 12 g. Na metabisulphite and 2·4 g. $Na_2SO_3 . 7H_2O$ in 100 ml. water).

*Standard:* 5 ml. phosphate standard (0·02 mg. P) *plus* 0·8 ml. ammonium molybdate and 0·2 ml. aminonaphtholsulphonic acid.

The colours are read in a photoelectric colorimeter with a red light filter.

*Calculation:*

Phosphatase units

$$= \frac{E \text{ of test} - (\text{control} + \text{blank})}{E \text{ of standard}} \times 0{\cdot}02 \times \frac{100}{0{\cdot}2} \times 3$$

$$= \frac{E \text{ of T} - (\text{C} + \text{B})}{E \text{ of standard}} \times 30.$$

Inorganic P (mg./100 ml.)

$$= \frac{E \text{ of control}}{E \text{ of standard}} \times 0{\cdot}02 \times \frac{100}{0{\cdot}2}.$$

An alternative micro procedure uses stannous chloride as the reducing agent for the phosphate estimations. *Test:* 1 ml. buffer, 1 ml. phenylphosphate, 0·1 ml. plasma, 15 min. at 37°, 7·9 ml. trichloroacetic acid (5 %). *Control:* 2 ml. water, 0·1 ml. plasma, 7·9 ml. trichloroacetic acid. *Blank:* 1 ml. buffer, 1 ml. phenylphosphate, 0·1 ml. water and 7·9 ml. trichloroacetic acid.

A *standard* (5 ml., containing 0·005 mg. P), and 5 ml. of each of the filtrates, are treated with 0·8 ml. of the 5 % molybdate in 15 % $H_2SO_4$ and 0·2 ml. of dilute stannous chloride (1 in 10 dilution, with 10 % $H_2SO_4$, of 10 % $SnCl_2$ in 5 N-HCl).

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**An Improved Micro Acid-digestion Tube.** By
R. Parsons and T. A. Webster. (*Postgraduate Medical School, London. W.* 12)

**Investigations on some Cellular Stain Reactions.** By
J. C. White. (*Department of Pathology, Postgraduate Medical School, London, W.* 12)

Using Susa-fixed smears of biopsied human marrow and liver, intracellular distribution of nucleic acids has been studied with the aid of ribonuclease and desoxyribonuclease, staining by basic dyes, pyronin and methyl green, and by the Feulgen reaction.

Ribonucleic acid stains only with the pyronin. Nuclear desoxyribonucleic acid stains with both pyronin and methyl green, the relative affinity for the two dyes being influenced by the fixative, this factor possibly modifying the degree of polymeriza-

tion of the acid (see Kurnick, 1949), as well as affecting nucleoprotein linkages.

After removal of both nucleic acids by combined nuclease action or by 4% trichloroacetic acid extraction at 90° C. for 15 min. (Schneider, 1945; Pollister & Ris, 1947), the cellular proteins are still substantially intact, and stain with acid dyes below pH 4 in similar fashion to controls.

Using a mixture of orange G and aniline blue 707 at pH 2–3, globin of erythrocytes and, to a lesser extent, intranuclear histones retain orange G, whereas aniline blue more slowly stains plasma proteins and cytoplasmic proteins, including ribonucleoproteins. Some intranuclear structures, including chromonema threads, periphery of nucleoli and also the chromosomes finally stain deeply with aniline blue, possibly due to their content of non-histone proteins (Mirsky, 1947; Pollister & Ris, 1947) or of chromosomin (Stedman & Stedman, 1947). Similar staining of fixed films of proteins in the dye mixture shows that globin and histone* retain orange G strongly for a considerable period, whereas with ribonucleoprotein and desoxyribonucleoprotein complex, the initial orange G staining is rapidly replaced by aniline blue, as in the cytological preparations.

Extraction of ribonucleoprotein and desoxyribonucleoprotein complex from liver cells, by 0·85% and molar sodium chloride solutions respectively (Mirsky & Pollister, 1946), can be demonstrated cytologically by these staining methods, but with marrow cells extraction is more difficult.

In developing erythroblasts, the cytoplasmic affinity for orange G seems to follow the degree of formation of the globin moiety of haemoglobin, and it is noteworthy that the benzidine test for peroxydatic activity of haemoglobin becomes increasingly positive in these cells, in parallel fashion. The benzidine is dissolved in methanol containing 2·5% of acetic acid, and is followed by ozonic ether. Under these conditions, the peroxidase reaction of granular leucocytes is minimal.

* I am grateful to Prof. J. N. Davidson for a sample of histone, and to Drs E. M. and H. M. Jope for globin.

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## Haldane Haemoglobin Determinations with the Photoelectric Colorimeter.

By E. J. King and Maryse Geiser. (*Postgraduate Medical School, London, W.* 12)

The Haldane carbon monoxide haemoglobin standard defined by the British Standards Institute, and characterized by Donaldson, Harding & Wright (1943), furnishes a readily procurable and easily checked reference for the determination of haemoglobin in blood. The ordinary Haldane technique, in which a measured quantity of blood is saturated with CO and then diluted with water in a small test tube until its colour appears to match that of the standard, may give very inaccurate results (Macfarlane, 1945; Macfarlane, King, Wootton & Gilchrist, 1948). But the carbon monoxide procedure is nevertheless capable of yielding figures which conform to the true haemoglobin content of the blood with a high degree of accuracy, provided the assessment of the colour is made photometrically and with precision (King, Gilchrist, Wootton, Donaldson, Sisson, Macfarlane, Jope, O'Brien, Peterson & Strangeways, 1948). By employing photoelectric colorimeters (EEL, Gallenkamp, Hilger, etc.) and microadaptors which fit the B.S.I. Haldane standard and test tube, we have obtained results, much more easily and quickly than is possible by the dilution technique, which agree well with the most accurate colorimetric procedures and with iron and gas analyses.

A Haldane standard and test tube, marked only at the 2 ml. mark (100%), were obtained from Messrs Hawksley and Son, and the factor for conversion to true haemoglobin percentages of the ratio of test to standard readings was obtained by submission of the standard and test tube to the National Physical Laboratory, Teddington.

Blood (0·02 ml.) is washed from a capillary pipette into about 1 ml. of water (containing 0·4% of concentrated ammonia solution) in the test tube, and is saturated with carbon monoxide or with coal gas. Water (0·4% ammonia) is added carefully to the mark and the mixture gently shaken. The readings (extinctions) are then taken, of the test and the standard, in the photoelectric colorimeter fitted with a bright spectrum yellow-green light filter (Ilford 625) and the microadaptor for the Haldane tubes. The ratio of the readings (Test/Standard)

is multiplied by 100 and by the N.P.L. factor to give the concentration of haemoglobin in percentage of Haldane normal, or by 14·8 × the factor to give the haemoglobin in grams per 100 ml. of blood.

Results obtained by this procedure agreed with macro carbon monoxide determinations with a coefficient of variation of 2·1 %; with cyanhaematin determinations, 2·2 %; with oxyhaemoglobin by the M.R.C. photometer (King, Wootton, Donaldson, Sisson & Macfarlane, 1948), 2·3 %; and with iron analyses, 2·5 %.

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## Increase in the Silicosis-producing Effects of Small Amounts of Quartz by Admixed Coal Dust.

By E. J. KING, S. C. RAY and C. V. HARRISON. (*Postgraduate Medical School, London, W.* 12)

## Determination of Iodine in Blood and Urine.

By R. FRASER and POPPY SCARLET. (*Postgraduate Medical School, London, W.* 12)