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REVIEW ARTICLE

THE CARDIOACTIVE GLYCOSIDES

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INTRODUCTION

THE impressive description given by William Withering in 1785 of the action of digitalis leaves upon the failing heart resulted in such a fundamental advance in the treatment of cardiac diseases that, even to-day, therapeutic measures are still based upon the experiences he recorded. In the intervening period, however, it has been possible to adapt the methods of treatment better to the various diseases of the heart and circulation than was originally the case.

Since the time of Withering there has been introduced into therapy a whole series of cardioactive drugs fundamentally similar in effect to the leaves of *Digitalis purpurea*, but differing, for example, in their rapidity and duration of action.

The chemical investigation of the drugs from which the cardioactive glycosides are obtained also dates back to the middle of the 19th century. Particularly in the last two decades, however, it has made such rapid strides that to-day the constitution of practically every important cardiac glycoside is known, including the details of its steric configuration.

In this article, an attempt will be made to characterise the more important cardiac glycosides but without pursuing the long trail which led to their isolation and to the elucidation of their chemical constitutions. The main difference between modern processes of isolation and the earlier methods employed lies in the fact that account is now taken of the great sensitivity of the cardiac glycosides to high temperatures, to acids or alkalis, and to enzymatic cleavage, etc. Not only digitoxin but all the other cardiac glycosides known prior to 1930, with the exception of ouabain and scillaren A, are in fact, artificial products arising as the result of enzymatic cleavage from substances of a higher sugar content originally present in the plant. This reveals one reason for the difference in action repeatedly observed by physicians between the use of the older pure substances on the one hand and of the crude drugs on the other.

THE SUGARS OF THE CARDIOACTIVE GLYCOSIDES

One of the main characteristics of glycosides is the fact that they contain one or more sugar residues. In the cardiac glycosides, the following sugars have so far been discovered.

CHO	CHO	CHO	CHO	CHO
	$-OCH_3$			H_3CO-
CH_2OH	CH_3	CH_3	CH_3	CH_3
D-Glucose	L-Thevetose	L-Rhamnose	L-Talomethylose	D-Digitalose

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CHO	CHO	CHO	CHO	CHO
	CH_2	CH_2	CH_2	CH_2
---	$-CH_3$	$-OR$ H_3CO-		$-OCH_3$
CH_2	CH_3	CH_3	CH_2	CH_3

D-Gulomethylose   L-Oleandrose   R = H: D-Digitoxose   D-Diginose   D-Sarmentose
R = CH₃: D-Cymarose

Apart from D-glucose, they are all desoxysugars, i.e., they contain 1 or 2 oxygen atoms fewer than the corresponding carbohydrate with 6 C atoms. While D-glucose and L-rhamnose are fairly widely distributed, the remaining sugars have so far been found only as components of cardiac glycosides.

For the sake of completion, we have also given the formulæ for L-oleandrose and D-diginose, although the glycosides derived from these sugars will not be discussed. The chemistry of the oleander glycosides has been known for a long time and has been described in a number of comprehensive publications. Diginose is the sugar obtained from the non-cardioactive diginin, a glycoside obtained from the leaves of *D. purpurea,* which would be out of place in this article. The constitutions and configurations of both these sugars have been confirmed by synthesis[1,2].

The sugar residue which, in the cardiac glycosides, may consist of a chain of up to 4 sugar molecules, is responsible for the water solubility of the glycoside on the one hand and, on the other, for its power of fixation to the heart muscle.

THE STEROID STRUCTURE OF THE AGLYCONES

The aglycones of the cardiac glycosides possess much more complicated structures than the sugar components. These genins are the real carriers of the specific action even though, on account of their insolubility in water and their low power of fixation to the heart muscle, they are of no importance therapeutically. In structure they are very closely related to the steroids and they belong to this same large class of substances which also includes the sterols, the bile acids, the sex hormones, the hormones of the suprarenal cortex and vitamin D.

Our own investigations on the cardiac glycosides began more than 25 years ago with squill, a cardioactive drug from the Mediterranean countries which was used as a remedy for dropsy by the ancient Egyptians. Since we began our studies, we have isolated and thoroughly investigated the main glycoside of squill, scillaren A[3,4]. Our investigations are being continued on a number of other glycosides which accompany scillaren A in squill, but which are present only in very small quantities. They are also cardioactive and some of them are very beautifully crystalline.

In connection with scillaren A, a brief description will be given of the simplest chemical conversion of this cardioactive glycoside, or of its

aglycone, scillaridin A, into a product of animal origin, allocholanic acid[5].
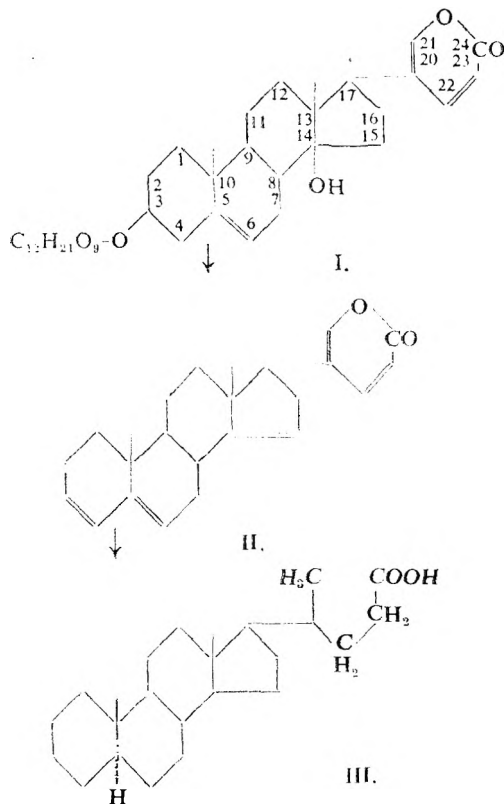
Schematic representation of the conversion of scillaren A into allocholanic acid.

Methyl alcoholic hydrochloric acid splits off the sugar residue and the tertiary hydroxyl group at C_{14} from scillaren A (I), resulting in the formation of two new double bonds, Δ^3 and Δ^{14}, and the production of anhydro-scillaridin A (II). On catalytic hydrogenation, all the double bonds of this substance are reduced and, at the same time, the lactone ring is opened by reduction so that a saturated desoxycarboxylic acid is formed. This has been shown to be identical with allocholanic acid (III) obtained by the method of Windaus[6] and Wieland[7] from hyodeoxycholic acid. Hence in the light of the already known constitution of allocholanic acid, direct conclusions may be drawn regarding the constitution of the aglycone obtained from the squill glycoside, since the sequence of the reactions was such as to avoid any severe attack upon the molecule, particularly any alteration in the structure of the carbon skeleton. The close relationship between the two classes of compounds, the aglycones of the cardiac glycosides on the one hand and the bile acids on the other, is thus demonstrated in a very simple manner. That the unsaturated lactone ring in scillaren A must be 6-membered, is proved beyond doubt

by this degradation. Wieland[5] has demonstrated that the same lactone ring is also present in the toad poisons.

In the structural formula of scillaren A, deduced from the conversion of anhydroscillaridin A into allocholanic acid, the position of the sugar molecule and of one of the neighbouring double bonds remained uncertain. The determination of the position of the sugar residue was made more difficult by the fact that when the sugar is split off from scillaren A the hydroxyl group to which it is attached is lost at the same time and a new double bond is formed. The point of attachment of the sugar chain which is built up from rhamnose and glucose and is known as scillabiose, was at first assumed to be position 5. It was possible[9], however, to establish with certainty that the sugar is attached to the hydroxyl group at C_3. In principle, the method was the same as that which led to the conversion of anhydroscillaridin A into allocholanic acid, the only difference being that the sequence of the reactions was altered, the hydrolysis not being performed until after the catalytic hydrogenation so that the hydroxyl group at C_3 was retained. In this way, it was possible to show that the acid obtained from scillaren A is identical with epi-allo-lithocholic acid (3β-hydroxy-allocholanic acid), prepared by the method of Wieland[7] from hyodeoxycholic acid, and, at the same time, to obtain proof that the hydroxyl group at C_3 carrying the sugar molecule bears the cis-configuration with respect to the methyl group at C_{10}.

In order to explain the fact that, in contrast to other rhamnosides, the sugar in scillaren A splits off easily with the formation of a conjugated system of double bonds, there must be a double bond in the neighbourhood of the hydroxyl group carrying the sugar. Hence, it follows that, by analogy with other unsaturated sterols, this ethylene linkage must be situated at $\Delta^{5,6}$. The formulæ of scillaridin A and anhydroscillaridin A corresponding to these conclusions are as follows:

IV.

II.
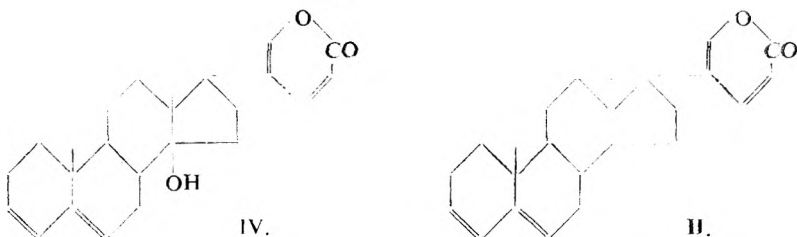
Although all the cardiac glycosides behave for the most part in a similar manner in their action on the heart, they exhibit certain differences which depend upon relatively small variations in the chemical structure. The following example illustrates how far the introduction of new groups results in changes in the type of action.

While rodents, particularly rats, are relatively speaking not very sensitive to glycosides of white squill and can tolerate comparatively large doses, they show an unusual sensitivity to one of the active principles of red squill. The red variety of squill contains a glycoside related to

scillaren A, scillirosid, which has only been prepared in a pure crystalline state and chemically identified in the last few years[10,11].

$C_{12}H_{21}O_9$ O   I.

$C_6H_{11}O_5-O$ V.
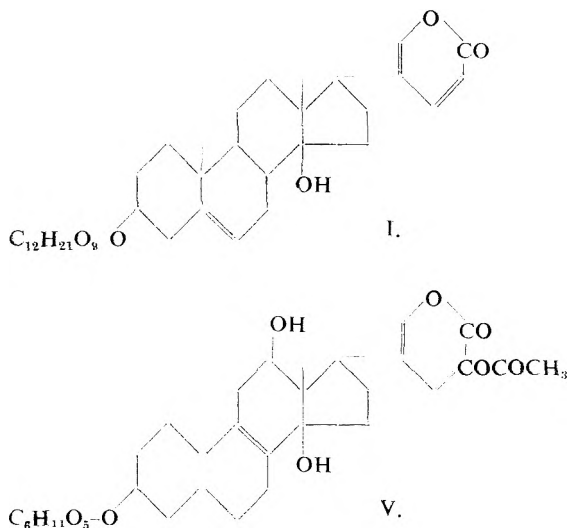
Constitutional formulæ of scillaren A and scillirosid

Scillirosid, like scillaren A, has a sugar residue in position 3, although this consists only of glucose. On the other hand, it contains, in addition, a hydroxyl group, probably in position 12, as well as a double bond which is very difficult to hydrogenate and a characteristic acetyl group on the lactone ring. This acetyl group is very likely responsible for the specific toxic action in rodents. 0.1 to 0.2 mg. of scillirosid given with the food is sufficient to kill an adult rat, while the same animal could tolerate 200 times this amount of the closely related scillaren A without harm.

A glycoside with a 6-membered lactone ring has also been discovered in a species of the Ranunculaceæ. Karrer[12] isolated from the rhizome of *Helleborus niger*, the Christmas rose, a glycoside, hellebrin, with a powerful cardiac action. To this compound, he attributed the following formula VI with an aldehyde group at C_{10} and the δ-lactone ring with two double bonds characteristic of scillaridin[13].

Glucose-O-R_1-O R_2

VI R_1 = Glucose, R_2 = H
VII R_1 = Rhamnose, R_2 = OH
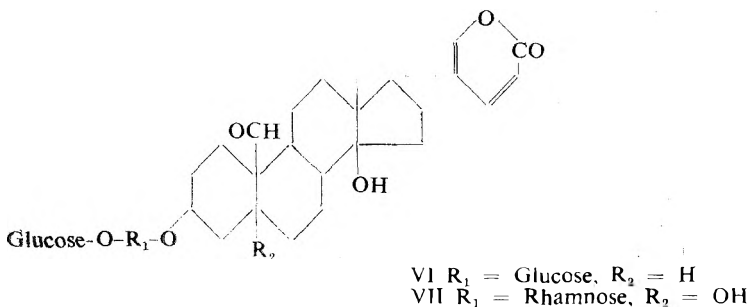
Reichstein and his co-workers[14,15], who demonstrated beyond doubt the presence of the aldehyde group, suggested formula VII. They found that hellebrin was decomposed by strophanthobiase to a mono-saccharide, desglucohellebrin. On cleavage by the method of Mannich, this yields L-rhamnose and two isomeric genins, which have not yet been further investigated. In this case, too. it is possible that the sugar chain is present in the form of scillabiose.

The aglycones of the cardioactive glycosides of the digitalis type and of strophanthus and certain other plants have a simple unsaturated 5-membered lactone ring in place of the 6-membered lactone ring with 2 double bonds present in scillaren A, scillirosid, hellebrin and the toad poisons. The remaining differences are connected with the peripheral structure of the molecule and depend upon the number and arrangement of the hydroxyl groups. Strophanthidin, the aglycone of k-strophanthin, like the aglycone of hellebrin, possesses the particular feature of an aldehyde group at C_{10}, whereas the other aglycones have a methyl group.

The reasoning which led to the above experimental proofs of the fine structure of the cardiac glycosides, was based upon the comprehensive knowledge which had been derived from the investigations into the steroids, and which, only about $1\frac{1}{2}$ decades ago, enabled the previously very incomplete conceptions of the chemical structure of the heart glycosides to be clarified.

ATTEMPTED SYNTHESES

That attempts to synthesise cardiac glycosides would soon follow and would lead to some interesting partial successes was to be expected. Thus Elderfield[16] and his co-workers, on the one hand, and Ruzicka[17] and co-workers on the other. starting from simple sterol deriva-tives and employing the Reformatsky reaction with bromacetic ester. succeeded in building up the 5-membered lactone ring char-acteristic of many aglycones. These experiments also showed that the previous assumption of a double bond in the β,γ-position to the carbonyl group of the lactone ring was incorrect and had to be replaced by a formu-lation in the α,β-position.

In all the structural formulæ given here, this alteration is already taken into account. The lactone ring was assumed by Jacobs and his co-workers to be β,γ-unsaturated, i.e., aldoenol lactone. since this position of the double bond appeared to agree particularly well with its reactions, and because. in particular, this structure was claimed to give the best

$$HC\text{---}CH_2$$
$$H_3C\text{-}C \qquad CO$$
$$\diagdown O \diagup$$
VIII.
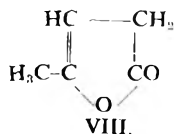
explanation for the colour reaction with sodium nitroprusside. the so-called Legal test[18]. It had, however. already been observed at that time that, on catalytic hydrogenation, the aglycones were not reduced to saturated deoxycarboxylic acids like other enol lactones. e.g.. α-angelica-

lactone[19] (VIII) or scillaren A[20], but that they took up only two atoms of hydrogen and that the double bond was reduced without opening of the lactone ring.

Moreover the aglycones with 5-membered lactone rings do not add on bromine on titration by Winkler's method[18]. That all aglycones so far investigated do, in fact, contain an α,β-unsaturated lactone ring, was shown by a thorough comparison with simple synthetic lactones and with lactones derived from steroids in which the position of the double bond had been established beyond question[16]. These comparisons concerned principally the behaviour of the lactone ring on cleavage with aqueous and with alcoholic alkali, the ultra-violet absorption and the colour reaction with potassium ferricyanide. The synthetic steroid lactones so far obtainable do not contain all the structural features of the natural cardiac aglycones. It was comparatively easy to prepare substances with a secondary hydroxyl group at C_3 and having the 5-membered lactone ring at C_{17} in the correct position. The introduction of the tertiary hydroxyl group at C_{14} has likewise been successfully achieved[21]. The synthesis of compounds which possess the configuration of the natural substances, both at C_{14} and at C_{17}, is being worked out.

THE CONFIGURATION OF THE AGLYCONES

This brings us to a further problem, the solution of which has received particular attention in the last few years, namely the configuration of the individual linkages in the aglycones. By precise evaluation of X-ray photographs, Bernal and Crowfoot[22] have shown that the natural steroids must possess a flat, relatively elongated molecule.

This necessitates that rings B and C should be united in the trans-configuration, both in the cholanic (IX) and in the allocholanic (X) series, which differ from one another in the configuration of C_5.

IX.   X.
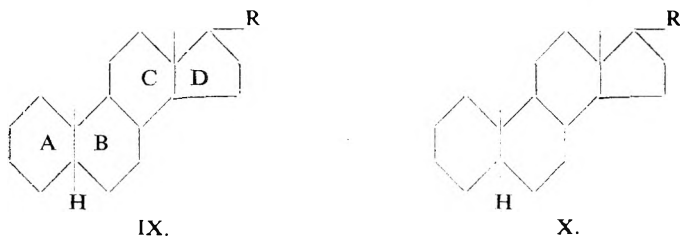
The allocation of the aglycones of the cardiac glycosides to the cholanic series follows from the degradation of, for example, digitoxigenin to ætio-cholanic acid. This degradation, as well as the numerous conversions which elucidated the connections between the individual aglycones, shows that the configuration of the asymmetric C-atoms 8, 9, 10, 13 and 17 in all these substances is the same as that present in the bile acids. We shall, therefore, discuss the steric relationships for those positions where particular isomerisms can occur. The two possible positions for substituents on asymmetric carbon atoms in the steroid skeleton will be denoted by the Greek letters α or β and indicated in the usual manner by dotted or by continuous valency lines. As point of reference, the C-atom in

position 10 will be selected and substituents which bear a *cis*-configuration with respect to the methyl group at this point will be defined as β-orientated.

The proof that epimerism occurs at C_3 based on the fact that cholesterol, for example, is precipitated by digitonin while certain derivatives are not, has been known for a long time. It has recently been shown, however, that not all 3β-hydroxysteroids are precipitated by digitonin, but that the precipitation can be prevented by certain substituents at other positions in the molecule. Thus, the aglycones of the cardioactive glycosides are themselves not precipitated by digitonin, and hence the configuration at C_3 had to be proved by chemical degradation in the manner shown above for scillaren A[9]. Hunziker and Reichstein[23] obtained epi-ætiolithocholic acid (3β-hydroxyætiocholanic acid, XII) from digitoxigenin (XI). The β-configuration of the 3-hydroxyl group

XI.

XII.

XIII.
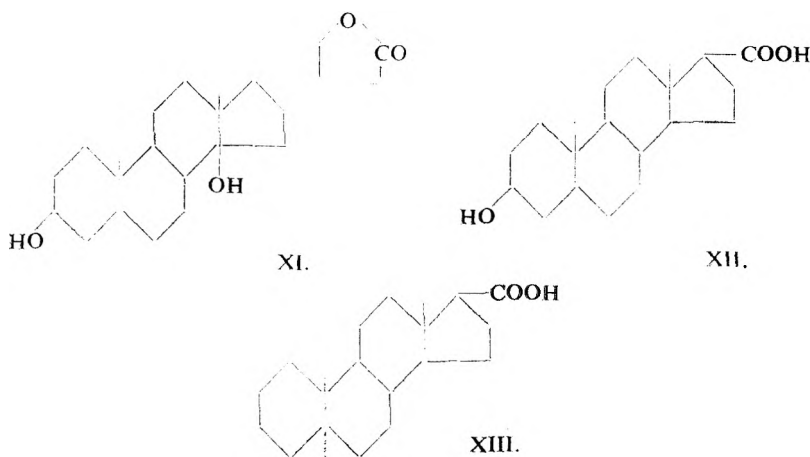
is also found in sarmentogenin[24] and in gitoxigenin, which has been shown, by a series of reactions which do not affect the configuration at C_3 to be related to digitoxigenin[25]. Uzarigenin[26] likewise possesses a β-orientated 3-hydroxyl group, since its anhydro derivative gives a precipitate with digitonin. The opposite configuration at C_3 is found in digoxigenin[27] as has been shown by degradation to the corresponding 12-epi-ætiodeoxycholic acid.

Strophanthidin and periplogenin must possess the same configuration at all asymmetric centres, since they can be transformed into identical derivatives by methods which do not affect the steric structure[28]. The hydroxyl groups at C_3 and C_5 must bear a *cis*-configuration to one another, because they can be esterified by thionyl chloride with the formation of a neutral sulphite[29]. The β-configuration of the two hydroxyl groups follows from the ring closure between the aldehyde group at C_{10} and the 3-hydroxyl group in the anhydro-strophanthidin derivatives[30], and from the fact that, if a carboxyl is added to the aldehyde group by cyanhydrin synthesis, lactone formation takes place with the 5-hydroxyl group[31]. The substituent at C_5 therefore has the same configuration as

the hydrogen atom in the other aglycones, and hence strophanthidin and periplogenin also belong to the cholanic series. Only uzarigenin possesses the allocholanic configuration, since it is degraded to ætio-allocholanic acid (XIII)[32].

The above-mentioned degradation of digoxigenin (XIV) to 12-epiætiodeoxycholic acid (XV) proves that the 12-hydroxyl group has the β-configuration.

XIV.   XV.
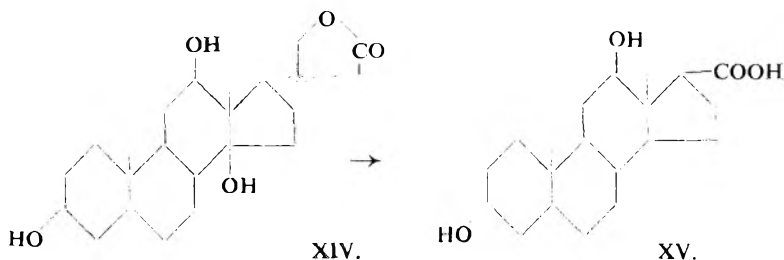
In scillirosid, the configuration at C_{12} must be the same as in digoxigenin since, in certain derivatives of iso-scillirosid, the presence of an oxide ring between C_{21} and C_{12} can be demonstrated, which would necessitate that the side-chain and the 12-hydroxyl group should possess the cis-configuration[33].

The 14-hydroxyl group which is common to all the aglycones of the cardiac glycosides must be formulated as β-orientated, since the oxide ring between C_{14} and C_{21} in the isocompounds of the aglycones can only be formed when the hydroxyl groups and the lactone side-chains possess the cis-configuration.

XVI.
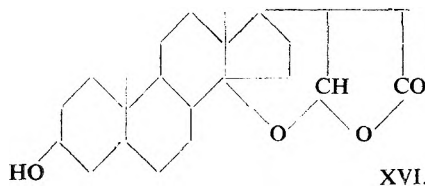
That the isomerisation of the aglycones by alkalis takes place without change of configuration at C_{17}, is proved by the degradation of digitoxigenin via iso-digitoxigenin (XVI) to ætiocholanic acid[34] and by degradation of anhydro-uzarigenin to allo-ætiocholanic acid[32]. Both acids possess β-orientated carboxyl groups. It may be mentioned here that Reichstein and his co-workers have demonstrated the β-configuration of the 14-hydroxyl groups in periplogenin[35] and in digitoxigenin[36] by another method, namely, by oxidative degradation to a 20-keto-21→14-lactone. Hence, in the cardiac aglycones, rings C and D are joined in the cis-position, in contrast to the bile acids and the steroids. For the cis-union of rings C and D Reichstein[37] has proposed the term 14-iso. Ruzicka the term 14-allo[38].

Under the influence of one of the enzymes found in strophanthus seeds, the glycoside cymarin undergoes a peculiar rearrangement[39], resulting in the formation of an isomeric physiologically inactive glycoside which

Jacobs and his co-workers have named allocymarin. On hydrolysis with acids, this yields allo-strophanthidin which is not isomerised by alkalis, but possesses the same functional groups as strophanthidin and must, therefore, be stereoisomeric with it. The systematic removal of the asymmetry at the oxygen-substituted asymmetric centres does not lead to identical derivatives from strophanthidin and allo-strophanthidin[40]. It must, therefore, be assumed that a change of configuration at C_{17} occurs on allomerisation. Thus, the side-chain takes up a *trans*-configuration with respect to the 14-hydroxyl group and isomerisation with the formation of an oxide ring can no longer take place. Periplocymarin (periplogenin-cymarosid) can also be allomerised by means of enzymes[41]. Reichstein[35] has shown that alloperiplogenin (XVIII) differs from periplogenin (XVII) only in the configuration at C_{17}, i.e., the allo-linkage carries an α-orientated side-chain. This may also be assumed to be the case for allo-strophanthidin.

XVII.   XVIII.

In the last few years, the introduction of simple sugars into natural aglycones and into synthetically prepared steroid lactones has been successfully accomplished, so that a whole series of partially synthetic glycosides are now available[42,43,44,45]. In addition, it has been shown that, in pharmacological experiments, synthetic substances obtained from natural aglycones by introduction of sugar residues often exhibit a high activity which, in certain cases, exceeds that of the natural glycosides. Up till now, however, no clinical results are available relating to the therapeutic activity and utility of these compounds. In the case of substances with synthetic lactone rings, the activity, although detectable, was only very slight. They lack certain structural features necessary for a high activity, such as, above all, certain steric relationships. This would almost seem to justify the old contention that Nature, in many cases, provides the physician with the remedy in its optimum form. In this connection, the extremely favourable properties of penicillin may be recalled.

The Sugar Components of the Genuine Glycosides

The close relationship which the various cardiac glycosides bear to one another is also manifest with regard to the position and composition of the sugar residue. In all the cardiac glycosides so far examined, this occupies the 3-position, where the aglycone is usually united with a

desoxy-sugar. This, in some cases, may in turn be combined with further desoxy-sugars and finally with 1 to 2 molecules of glucose. The glucose is readily split off by enzyme action and, for this reason, as already mentioned, the previously isolated glycosides such as digitoxin and gitoxin, or even digoxin from *Digitalis lanata,* lacked the terminal glucose, whereas, in the genuine glycosides obtained by excluding enzyme action, the glucose is present. The simplest example of a genuine cardiac glycoside is once more provided by scillaren A the composition of which is depicted schematically in the following diagram:

<div align="center">

SCILLAREN A

OH

scillabiose

O—rhamnose-β_1, D-glucose

scillaren A

XIX.

</div>
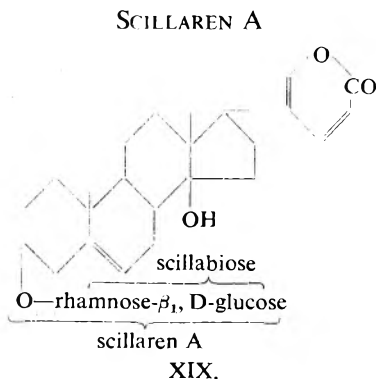
The sugar residue is situated at C_3 and is composed of rhamnose and glucose. Acids break the linkage with the aglycone and split off scillabiose[3,4], while scillarenase, an enzyme accompanying the glycoside in squill, breaks the linkage between rhamnose and glucose[46]. Thus, by means of this enzyme, and only in this way, a step-wise degradation may be carried out and the intermediate product proscillaridin A, a beautifully crystalline glycoside, obtained. Proscillaridin A has too low a solubility for therapeutic purposes, the only sugar present being rhamnose.

In the case of the genuine cardiac glycosides of the digitalis type, the conditions are somewhat more complicated. We had already learnt in our experiments with squill to prevent the action of the enzyme responsible for splitting off glucose. When we applied this process to the extracts of the glycosides of *Digitalis purpurea,* we obtained, instead of the already well-known crystalline glycosides digitoxin and gitoxin, amorphous but likewise very active products. The failure to crystallise was a great obstacle to the complete purification and identification of the genuine glycosides of *D. purpurea.* Only after we had first obtained experience with the glycosides of *D. lanata*[47] were we able to complete the investigations of the purpurea glycosides[48]. From *D. lanata,* by a process excluding enzymatic action, it was possible to obtain a crystalline glycosidal preparation which soon proved to be a mixture of 3 very different isomorphous glycosides which we designated digilanid A, digilanid B and digilanid C.

The marked differences in the distribution of the individual components between chloroform and aqueous methyl alcohol enabled the total

digilanid preparation to be resolved into the homogeneous components by repeated systematic partition between the two solvents. Separation of the components by fractional crystallisation is rendered very difficult by the isomorphous nature of the crystals.

The space at our disposal is not sufficient for a detailed description of the three digilanids A, B and C, and the products of their step-wise degradation. We shall, therefore, confine ourselves to digilanid A. Analogous conditions exist for digilanids B and C. The sugar chain is identical in all three digilanids.

SCHEME FOR DIGILANID A
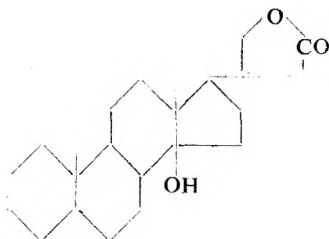
O-digitoxose–digitoxose–acetyldigitoxose-β, D-glucose
XX.

The formula shows the unsaturated lactone ring in position 17 and 2 hydroxyl groups in positions 3 and 14, the one in position 3 carrying the sugar chain. The latter consists of 3 molecules of digitoxose and a terminal glucose. The third molecule of digitoxose also carries an acetyl group which is characteristic for the digilanids and is responsible for the isomorphism. If it is removed, the A and B components lose their power of crystallisation[47]. They yield amorphous substances which have proved to be identical with the likewise amorphous genuine glycoside of *D. purpurea*.[48] These, in turn, by enzymatic removal of the terminal glucose, are converted into digitoxin or gitoxin. Thus the relationship between the glycosides of *D. lanata* and *D. purpurea* is clearly shown. The difference between the genuine glycosides of the two plants consists therefore in the presence of an acetyl group in the lanata glycosides.

For digilanid C, which has a composition very similar to that of digilanid A, there is no corresponding glycoside in *D. purpurea*. The digilanid C-structure has so far been found only in *D. lanata* and, as Mannich[50] has shown, in *D. orientalis*, a variety very similar to lanata which is found in Asia Minor.

The best example of the step-wise degradation of a genuine glycoside is provided by strophanthin or k-strophanthosid as we designate the crystalline active principle of the seeds of *Strophanthus kombé*. Jacobs[51] in New York had already isolated in small quantities from this drug two crystalline glycosides: cymarin, consisting of the aglycone strophanthidin and cymarose, and k-strophanthin-β, which contains one glucose molecule more than cymarin. The main part of the glycosides

isolated from this drug remained amorphous but, in the form of strophanthin, a greatly purified preparation, they have been widely used for many years. In this connection, the pioneer work of Fraenkel[52] deserves especial mention.

By employing a special process utilising the peracetyl derivative (heptacetyl) we have succeeded in converting the main portion of the total glycosidal preparation from strophanthus seeds into a crystalline and pure form and have introduced it into therapy as k-strophanthosid, known commercially as "Strophosid"[53]. The following scheme shows clearly the composition and the step-wise degradation by which all the cleavage products could be isolated and characterised.

K-STROPHANTHOSID

strophanthotriose

O-Cymarose-β, D-glucose-α,D-glucose

strophanthobiose
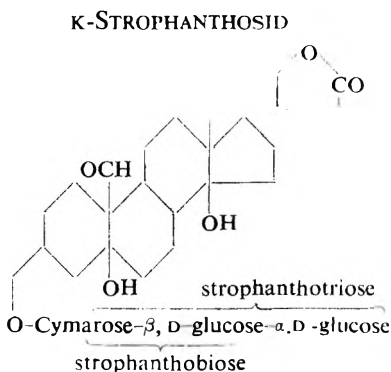
XXI.

The aglycone, strophanthidin, possesses hydroxyl groups at C_3, C_5 and C_{14}. At C_{17} the 5-membered unsaturated lactone ring is attached and at C_{10} the aldehyde group characteristic of strophanthidin. As in the case of the other cardioactive glycosides of the digitalis group, the oxygen atom at C_3 forms the bridge to the sugar chain, which, in k-strophanthosid, consists of cymarose and two molecules of glucose. If the terminal glucose is split off by enzymatic hydrolysis, k-strophanthin-β is produced. By splitting off a further molecule of glucose with the specific enzyme strophanthobiase, k-strophanthin-β is converted into cymarin which consists only of the aglycone strophanthidin and cymarose. The linkage between the aglycone and the sugar chain is broken by acid. From k-strophanthosid, a sugar, strophanthotriose, is obtained which consists of one molecule of cymarose, and two molecules of glucose. Cleavage of k-strophanthin-β leads to strophanthobiose, composed of cymarose and glucose. Acid cleavage of cymarin yields the aglycone and cymarose.

The example of k-strophanthosid enables an exceptionally good insight to be obtained into the fine structure of the glycosidal linkages and, hence, into the nature of the enzymes which are responsible for sugar cleavage and are specific for the cardioactive glycosides. As the nature of these enzymes was originally unknown, nothing could be said regarding the configuration of the linkages between the sugars. It was found that a well-known enzyme, the α-glucosidase of yeast, was capable of splitting the linkage between the two glucose residues of k-strophanthosid, so that,

by means of an enzyme obtained from an entirely different source, the genuine glycoside could be converted into k-strophanthin-β. Thus, the α-glucosidal nature of the linkage with the terminal sugar residue was established. So long as the sugar residue remains attached to the aglycone, however, the linkage between glucose and cymarose can only be broken with the specific enzyme strophanthobiase.

Once it is separated from the aglycone with acid, the disaccharide strophanthobiose can be split by means of the well-known β-glucosidase emulsin[54]. The linkage between cymarose and glucose is therefore of a β-glucosidal nature and strophanthobiase, as far as its specific power of hydrolysing glycosides is concerned, must be classed with the β-glucosidases.

Thus, in k-strophanthosid, the inner glucose molecule has the β-form, while the outer has the α-form. k-Strophanthosid is the first heterosaccharide to be found in nature in which an α-glucosidal configuration has been established with certainty and it is therefore also the first natural heterosaccharide containing both an α- and a β-glucose residue.

In an exactly similar manner to that used in the case of k-strophanthosid, it has been possible to show that, in the other cardiac glycosides, the linkage between the terminal glucose and the desoxysugar is also of a β-glucosidal nature and hence that the specific enzymes digilanidase, digipurpidase and scillarenase belong to the β-glucosidases[54].

OTHER GLYCOSIDES OF STROPHANTHUS SPECIES

The cardiac glycosides so far discussed in no way exhaust the multiplicity of this class of substances. In addition to the digitalis and strophanthus types and to *Scilla maritima,* many other plants are known which contain cardiac glycosides. Some of these possess aglycones or sugars which were not previously known. Some of them, however, are only variations of already known cardiac glycosides in the sense that both the aglycone and the sugar have already been found in the glycosides of digitalis or strophanthus though here they are differently coupled. Moreover, most varieties of strophanthus contain, in addition to the strophanthidin glycosides a number of other cardioactive glycosides, the preparation of which in a pure, intact form has, however, not yet been accomplished. On the other hand, after enzymatic hydrolysis, it has been possible to prepare the glucose-free glycosides which correspond in composition to cymarin (XXII). The variations may be seen from the following formula .

They differ from cymarin principally in the stage of oxidation of the C-atom 19 in the genin. The glycosides of periplogenin, periplocymarin (XXIII) (19-desoxo-strophanthidin) and emicymarin (XXIV), have no oxygen atom in this position. The former contains the sugar cymarose, the latter the sugar digitalose first found in digitalinum verum, the seed glycoside of *Digitalis purpurea.* Cymarol (XXV) possesses an alcoholic hydroxyl group at C_{19}. Its aglycone, k-strophanthidol, can be obtained from strophanthidin by reduction of the aldehyde group[55]. In cymarol, the hydroxyl group at C_3 is united to cymarose by a glycosidal linkage.
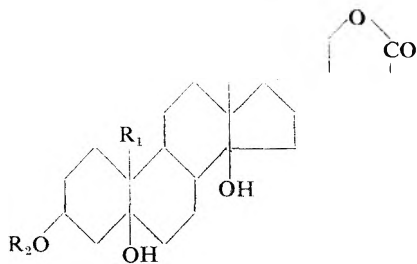
XXII R_1 = CHO, R_2 = cymarose
XXIII R_1 = CH_3, R_2 = cymarose
XXIV R_1 = CH_3, R_2 = digitalose
XXV R_1 = CH_2OH, R_2 = cymarose

Cymarol can be shown to be present in all strophanthus seeds which contain the glycosides of periplogenin and strophanthidin. These glycosides are found in the seeds of *Strophanthus kombé*[56], *S. eminii*[57], *S. nicholsoni*[57] and *S. hispidus*[57]. However, the botanical differentiation of strophanthus seeds is not always easy and the results of examinations of hispidus seeds in particular are for this reason rather uncertain.

The glycosides of the seeds of *S. gratus* and *S. sarmentosus* differ fundamentally from the glycosides described above. Strophanthidin, k-strophanthidol and periplogenin glycosides cannot be detected in the seeds of either of these varieties. In gratus seeds, only one glycoside, g-strophanthin, has been found and this has been shown to be identical with ouabain isolated from the bark and wood of *Acocanthera ouabaio*, an African plant of the Family Apocynaceae[59]. The sugar component of ouabain, the name by which g-strophanthin is more usually known, consists of a single molecule of rhamnose. Even under conditions which avoid enzymatic cleavage, only ouabain is obtained from gratus seeds[53]. k-Strophanthin-β remains intact in contact with gratus seeds, from which it may be concluded that these seeds do not contain a glucosidase and probably no glucose-containing glycoside. Being a rhamnosid, ouabain is very difficult to split. The hydrolytic cleavage was first accomplished
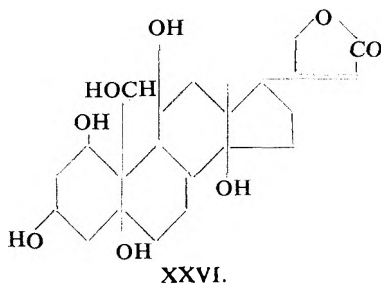
XXVI.

by Mannich and Siewert[60], who proposed for ouabagenin a complete structural formula (XXVI) with a hydroxyl group at C_5.

Since, however, under certain conditions ouabain yields a heptacetate, its genin must contain four acylable hydroxyl groups in addition to

those involved in the glycosidal linkages. This argues against a tertiary hydroxyl group at C_5, unless it is assumed that, in ouabain, in contrast to other cardiac glycosides, the sugar is attached at this point. By a number of ring closures, Mannich has provided good evidence for the positions of the other hydroxyl groups. As regards the configuration of the asymmetric C-atoms to which the oxygens are attached, it can only be said that all the hydroxyl groups are *cis*-orientated to one another.

The seeds of *Strophanthus sarmentosus* contain a mixture of glycosides none of which has yet been prepared in its native state, although three glycosides, sarmentocymarin[61] and sarmentosids A and B[62] have so far been obtained after enzymatic removal of glucose. Sarmentocymarin, an already well-known glycoside, yields, on acid hydrolysis, sarmentogenin and the methyl ether of a 2-desoxy-methylpentose, sarmentose. Although the configuration of this sugar has not yet been established with absolute certainty, on page 850 we have assigned to it the configuration of 2-desoxy-D-idomethylose (2-desoxy-D-gulomethylose). On the assumption that sarmentose possesses a straight C-chain, this is the only possible configuration, since, from each of three of the four pairs of isomers theoretically possible, at least one partner is known and sarmentose is neither identical nor enantiomorphous with any of these. The structure of sarmentogenin has been established by degradation to 3β-11α-diacetoxy-ætiocholanic acid methyl ester[24] as that depicted in formula XXVII.
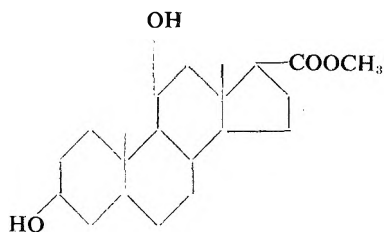
XXVII.

In the case of sarmentosids A and B only the sugar components are known. Sarmentosid A contains L-talomethylose[63], a sugar which had not previously been found in a natural product. Sarmentosid B contains the sugars D-glucose and digitalose[62]. A striking feature here is that the glucosidal linkage is resistant to the attack of the specific enzyme.

CARDIAC GLYCOSIDES OF UNOFFICIAL DRUGS

Of the remaining drugs which contain cardiac glycosides, only those whose active principles have been chemically investigated during the last 10 years will be discussed. First of all, mention should be made of *Convallaria majalis*. Convallatoxin, the glycoside contained in its leaves and flowers, was isolated and described a long time ago by W. Karrer[64]. Reichstein and Katz[65] were, however, the first to elucidate its constitution, using the method of Mannich which hydrolysed it to strophanthidin and L-rhamnose. The seeds of *C. majalis* also contain cardiac glycosides. One of these was isolated in a pure state by Schmutz

and Reichstein[66] and given the name convallosid. It is split by strophanthobiase to yield convallatoxin and D-glucose. Thus, its sugar structure corresponds to that of scillaren A. Nevertheless, it has not yet been possible to split off the sugar chain as a whole, but this would presumably be in the form of scillabiose. Convallosid is very probably the genuine glycoside of the seeds of C. majalis.

The roots of Adenium somalense, a plant of the Family Apocynaceae, together with varieties of strophanthus and acocanthera, are used by certain native African tribes, particularly in Kenya, for the preparation of arrow poisons. It has been found that they contain a crystalline glycoside to which Hartmann and Schlittler[67] have given the name somalin. On acid hydrolysis, somalin is decomposed into digitoxigenin and cymarose and is therefore so simple in structure that, apart from the configuration of the glycosidal linkage, all the structural details can be deduced merely by hydrolytic cleavage.

The aglycone of the glycosides present in the nuts of Thevetia neriifolia has been identified as digitoxigenin[68]. At the same time, it was shown that thevetin is decomposed both by the drug enzymes[69,70] and by strophanthobiase[70] and the digestive enzymes of snails[71], yielding the glucose-free neriifolin. The isolation of acetylneriifolin suggests that the genuine glycoside is present as an acetyl derivative of thevetin.

Similar glycosides have been found in the nuts of Cerbera odollam. Of these cerberosid[72], which is assumed to be the genuine glycoside, is converted by enzymatic degradation [73,74] into neriifolin or monoacetyl neriifolin (cerberin)[75,76].

Thevetose is also the characteristic desoxysugar of the glycosides contained in the nuts of Tanghinia venenifera, another member of the Apocynaceae[77]. Frèrejacque and Hasenfratz were able, after isolating several glucose-free glycosides from the amorphous, acetyl-containing tanghinosid, to prepare gentiobiose, so that here, too, the nature of the sugar residue has been to a large extent cleared up[78].

The leaves of Adonis vernalis (Ranunculaceae) contain at least two glycosides, cymarin and adonitoxin[79], which, together, may be considered to be mainly responsible for the pharmacodynamic action of this drug. Adonitoxin has been further investigated by Katz and Reichstein. It contains the sugar component L-rhamnose united with an aglycone which is isomeric, but certainly not identical with strophanthidin. This aglycone, likewise, possesses an aldehyde group and the simply unsaturated γ-lactone ring characteristic of the genins of digitalis and strophanthus glycosides, but, in contrast to strophanthidin, it has only one tertiary and two acylable hydroxyl groups.

From the seeds of Cheiranthus cheiri (Goldlack), a cardioactive glycoside cheirotoxin can be isolated[80]. On cleavage by the method of Mannich, this yields strophanthidin and a sugar syrup from which phenyl glucosazone can be obtained. In addition to glucose, however, cheirotoxin contains a pentose, the D-lyxose[81] which is the first pentose found as a component of cardiac glycosides.

The sap of *Antiaris toxicaria* provides two glycosides α- and β-antiarin which, according to Kiliani[82], differ only in their sugar components. β-antiarin contains L-rhamnose, α-antiarin the sugar of D-gulomethylose[83], which is found here for the first time as a constituent of a natural product. Reichstein and co-workers were the first to prepare intact antiarigenin, to which they assigned the formula XXVIII with a secondary non-acylable hydroxyl group, the position of which has not yet been determined.
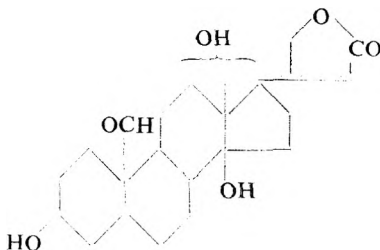
XXVIII.

Evonosid, the glycoside from the seeds of *Euonymus europæus*, contains a sugar chain consisting of two molecules of glucose and one of rhamnose. On treatment with strophanthobiase, step-wise degradation takes place as a result of which the intermediate product evobiosid and the end-product evomonosid are formed[84].

The seeds of practically all the known species of *Coronilla* belonging to the section Eucoronilla contain cardioactive glycosides. We have investigated the seeds of *Coronilla glauca* more exactly[85]. Cautious extraction yielded an amorphous mixture which it was impossible to separate. On acid hydrolysis, the genin portion completely resinified while, in the sugar portion, only glucose was found. From the yield of the latter it was concluded that the genuine glycosides were built up from the genin and, on the average, two molecules of sugar. Enzymatic degradation of the glucosidal mixture with the specific enzyme of Coronilla seeds led direct to the aglycones, a result which had previously not been obtained with any other hydrolytic enzyme specific for cardiac glycosides.

From the mixture of aglycones, in addition to a considerable quantity of a furocumarin, it was possible to isolate in a crystalline form four different compounds with the carbon skeleton of the genins of the cardiac glycosides. These are:

Allo-glaucotoxigenin $C_{23}H_{32}O_6$
Corotoxigenin $C_{23}H_{32}O_5$
Coroglaucigenin $C_{23}H_{34}O_5$
Glaucorigenin $C_{23}H_{32}O_3$

Allo-glaucotoxigenin, corotoxigenin and glaucorigenin contain an aldehyde group at C_{10} of the steroid skeleton; coroglaucigenin has a methyl group in this position. Allo-glaucotoxigenin is inactive; it represents the

allo form, which probably arises through the action of an allomerising enzyme present in the seeds.

The above review shows that the investigations of the composition, structure and configuration of the individual cardio-active glycosides and on their synthesis have reached impressive proportions. Moreover, it emphasises the important part played by enzymatic studies in this field of research.

The chemical investigations on the cardiac glycosides which have so far been made have provided a fairly clear and complete picture, and have laid the foundations for the pharmacological investigations and for the clinical application and differentiation of the various glycosides. Nevertheless, it will be well worth while, particularly in view of the rapid increase in diseases of the heart and circulation, to expand still further our knowledge of the completed and improved therapeutic armamentarium, to which the glycosides known to-day have certainly contributed. Only on the basis of long experience will it be possible to establish rules which will make it easy, in each individual case, to select the best from the good preparations.

If data on the pharmacological action and the therapeutic application of cardiac glycosides have not been given in this article, it is because it would be out of place here to discuss the treatment of heart diseases. It should, however, be stressed that in this field therapy cannot be carried out according to any pre-arranged scheme, but that it still requires, more now in fact than ever, the whole art of the experienced physician.

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RESEARCH PAPERS

SODIUM AND CALCIUM GLYCEROPHOSPHATES
A Survey

BY J. S. TOAL AND J. I. PHILLIPS

THE object of this paper is to draw attention to the unsatisfactory position now existing in which commercial supplies of glycerophosphates, although issued as complying with the standards of the British Pharmaceutical Codex, do not actually satisfy the full requirements of the monographs. In order to clarify this position the authors of the present paper produced some pure salts, following factory practice, and investigated them. Published work by other investigators in this subject may be referred to from the bibliography given at the end of the paper[1,2,3,4,5,6,7,8].

DIFFERENTIATION BETWEEN THE ISOMERS

In order to differentiate between the α and β-salts of glycerophosphoric acid an efficient method of assay was required. This was found in the Malaprade reaction of periodic acid on vic. glycols for the estimation of the α-isomer. This method was first applied by Fleury and Paris[9] to the assay of α-glycerophosphates, using arsenious acid for determining the excess of periodic acid, and was later adopted by Pyman and Stevenson[8]. But the present authors found the arsenious acid method somewhat difficult to manipulate, and they prefer the direct procedure of acidifying in the presence of excess of potassium iodide and determining the amount of iodine lost in the reduction of the periodic acid to iodic acid, in comparison with a standard blank. Both methods gave results in complete agreement.

0.3 g. of the crystalline sodium glycerophosphate (or 0.15g. if the α content exceds 50 per cent.) is weighed, placed in a stoppered 250-ml. conical flask and dissolved in a minimum amount of water; 25 ml. of a periodic acid solution (prepared by dissolving 3.674 g. of $Na_3H_2IO_6$ in 37.5 ml. of N sulphuric acid and diluting to 500 ml.) is added, the flask is swirled and set aside for 10 minutes. The reaction that takes place is represented by the equation—

$$CH_2 \cdot O \cdot PO_3Na_2$$
$$|$$
$$CHOH \quad + KIO_4 = KIO_3 + CH_2 \cdot O \cdot PO_3Na_2 + HCHO + H_2O$$
$$| \qquad\qquad\qquad\qquad\qquad |$$
$$CH_2OH \qquad\qquad\qquad\qquad CHO$$
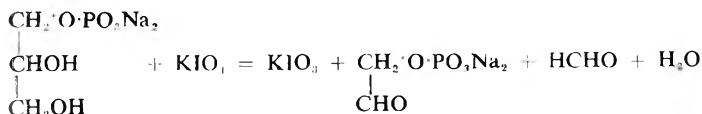
After standing, 1 g. of sodium bicarbonate is added to the flask followed by 5 g. of potassium iodide and 10 ml. of dilute hydrochloric acid. The carbon dioxide evolved is allowed to displace the air, the flask is stoppered, and set aside for 10 minutes, and the solution finally titrated with 0.1N sodium thiosulphate. A blank must be carried out in a

similar manner and the difference between the two titrations noted. The reactions that take place on acidification are—

$$KIO_4 + 8HI = KI + 4H_2O + 4I_2$$
$$KIO_3 + 6HI = KI + 3H_2O + 3I_2$$

Thus when 1 molecule of potassium periodate is reduced to potassium iodate a loss of iodine is shown in the final titration.

Hence, from the first equation above, 1 gram-molecule of sodium glycerophosphate is equivalent to 1 gram-molecule of potassium periodate, or 1 gram-molecule of iodine, or 2 litres N/1 thiosulphate. Thus each ml. of 0·1N thiosulphate in the difference noted is equivalent to 0·0162 g. of the α-salt $C_3H_7O_6PNa_2,6H_2O$.

In a mixture of the isomers, the amount of α-sodium glycerophosphate can be found by the above method, and the amount of the β-salt determined by titrating the total glycerophosphate and deducting from that result the titre due to the α-salt. The indicator in the titration should be methyl yellow, which gives a much sharper end point than methyl orange. At this stage of the work it had been assumed that the samples crystallising from the liquors were pure mixtures of α and β-isomers and contained no other titratable matter.

Samples of calcium glycerophosphate were assayed in a similar manner, taking 0·125 g. of the salt, dissolving this directly in 25 ml. of the periodic solution and completing the titration in the manner already described; the difference between the titration of the blank and the test being recorded, 1 ml. of 0·1N thiosulphate being equivalent to 0·0105 g. of anhydrous calcium glycerophosphate.

When analysing sodium glycerophosphate liquors from the original combination, and also the mother liquors from which crops of crystals have been taken, it is essential that any free glycerin shall be completely removed, by repeated extraction with alcohol, because even traces of glycerin give rise to error in the assay for the α-salt.

PREPARATION OF PURE α AND β-SODIUM GLYCEROPHOSPHATE

Crude sodium glycerophosphate was prepared by combining, under vacuum, two equivalents of glycerin with one equivalent of sodium acid phosphate and then hydrolysing the resultant diglyceryl ester with sodium hydroxide. The glycerin was removed by extraction with alcohol and, after separation, the alcohol that remained was expelled by evaporation. Samples from three bulk batches were adjusted to contain 50 per cent. w/w of $C_3H_7O_6PNa_25\frac{1}{2}H_2O$ and the solution analysed. The results are shown in Table I.

TABLE I

Batch					Sp. gr. at 15·5°C.	Assay by titration	Assay by ignition	Residue at 150°C.	α-isomer (6H_2O)
						per cent.	per cent.	per cent.	per cent.
A ...	...	...	...	...	1·274	50·0	50·7	35·4 (theory 34·3)	23·1
B ...	...	...	...	...	1·277	49·5	50·6	35·4	21·0
C	...	...	...	...	1·277	50·0	50·7	36·3	22·0

These figures show the general constancy of the combination.

The bulked solution of these three batches was evaporated and adjusted to a strength of 70 per cent. w/w of $C_3H_7O_6PNa_2,5\frac{1}{2}H_2O$ and allowed to crystallise during several days. The crystals were removed and washed with alcohol. The mother liquor was again set to obtain further crystals and this procedure repeated until six different crops had been collected. The liquor from the sixth crop was uncrystallisable.

These six crops of crystals, after washing with alcohol to ensure the removal of any adherent glycerin, were assayed for their α β-content. The results are shown in Table II.

TABLE II

Crop							α- salt ($6H_2O$)	β-salt ($5H_2O$)	Total
							per cent.	per cent.	per cent.
1 ...	...	...	...	...	...	...	13·9	85·2	99·1
2 ...	...	...	...	...	...	...	71·0	30·9	101·9
3 ...	...	...	...	...	...	...	91·5	7·85	99·35
4 ...	...	...	...	...	...	...	28·5	72·9	101·4
5 ...	...	...	...	...	...	...	71·0	30·1	101·1
6 ...	...	...	...	...	...	...	42·0	57·8	99·8

Each of the six samples satisfied all the chemical requirements of the B.P.C., but all failed to comply with the opening definition that " sodium glycerophosphate is the sodium salt of β-glycerophosphoric acid."

Table II shows that the first crop of crystals contain the highest proportion of the β-isomer, whereas the third crop consisted almost entirely of the α-variety. These two crops of crystals were taken separately and twice recrystallised; finally separated from their mother liquor by centrifuging then washed with alcohol and dried at 60°C.

The analysis of these recrystallised preparations (given in Table III) shows that two pure isomers were obtained, the α-isomer containing 6 molecules of water of hydration and the β-isomer 5 molecules.

TABLE III

Tests						1st crop (twice recrystallised) β-isomer	3rd crop (twice recrystallised) α-isomer
						per cent.	per cent.
Loss at 150°C. ...	...	...	...	...		29·75 (theory 29·42)	33·25 (theory 33·34)
Assay (titration) ...	...	...	...	...		99·30 $C_3H_7O_6PNa_2,5H_2O$	99·50 $C_3H_7O_6PNa_2,6H_2O$
Assay (gravimetric)	...	...	...	...		99·20 " "	99·20 " "
α-isomer ...	...	...	...			absent	99·00 " "
Sp. gr. of 50 per cent. w/w solution	...	...				—	1·271 at 15·5°C.

A saturated solution of the α-isomer contained the equivalent of 63·5 per cent. w/w of $C_3H_7O_6PNa_2,6H_2O$ and a saturated solution of the β-isomer contained 45·5 per cent. w/w of $C_3H_7O_6PNa_2,5H_2O$.

The crystals of the α-isomer were hard and of a semitransparent nature, quite distinct from those of the β-isomer, which were fragile and of needle-like appearance.

The uncrystallisable liquor, which represented 10 per cent. of the original combination, was diluted with sufficient water to keep any α and β-calcium glycerophosphate in solution and then treated with a solution of calcium acetate. The precipitate that formed was collected

and analysis indicated its being a di-ester $CHOH(CH_2.O.PO_3Ca)_2$: the figures based on material dried at 150°C. are given in Table IV.

This calcium salt did not reduce periodic acid solution, and therefore was not a vic. glycol. An approximate computation showed that 100 g. of presumptive sodium glycerophosphate in the uncrystallisable mother

TABLE IV

	Calcium	Residue on ignition	Phosphorus
	per cent.	per cent.	per cent.
For suggested formula	24·4	77·5	18·50
For the precipitate	25·5	78·1	18·60

liquor consisted of 55 parts of di-ester and 45 parts of true sodium glycerophosphate; yet, when adjusted to a liquor containing 50 per cent. of apparent sodium glycerophosphate $(5\frac{1}{2}H_2O)$ as estimated by titration, it agreed with the standards set in the B.P.C.

CALCIUM GLYCEROPHOSPHATE

Calcium salts were prepared from solutions of the pure α and β-salts of sodium glycerophosphate.

With the β-salt, the method employed was that of treating the solution with an excess of 30 per cent. w/v aqueous solution of calcium acetate, and following with an excess of alcohol sufficient to throw down the calcium glycerophosphate. This was collected, washed free from calcium and sodium acetates by means of alcohol, dried and then examined. It held one molecule of water of hydration $(1H_2O)$ and was soluble to the extent of 0·96 g. of anhydrous material in 100 ml. of solution at room temperature.

With the α-salt, two methods of preparation were employed: one precisely as just described for the β-salt; the other, that of using more concentrated solutions (without the use of alcohol) and allowing the calcium glycerophosphate to crystallise from the supersaturated condition. In both cases the material was collected, washed with alcohol, dried and analysed. The precipitate effected by means of alcohol contained 2 molecules of water of hydration $(2H_2O)$, and that depositing from the supersaturated solution only 1 molecule $(1H_2O)$. The analytical figures for the 3 salts are given in Table V.

SOLUBILITY OF THE TWO α-SALTS IN WATER

Each salt was treated with distilled water, leaving a little of the salt undissolved. After about an hour's digestion 50 ml. of solution was withdrawn and titrated and the amount of calcium glycerophosphate in solution calculated. The solution of the $2H_2O$ salt contained 4·3 per cent. of the anhydrous salt and that of the $1H_2O$ salt 1·17 per cent. The suspensions were set aside for 3 days, with occasional shaking, and then again tested. The amount of $2H_2O$ salt in solution had dropped to 4·0 per cent., whilst that of the $1H_2O$ salt remained at 1·17 per cent. The

mixture containing the $2H_2O$ salt was now tested periodically until constant solubility was attained. This occurred at 1·26 per cent. of the anhydrous material after about 4 weeks' digestion at from 20° to 25°C.; considerable precipitation having taken place. The residuum of this digest was collected, washed with alcohol, dried and examined: it consisted of α-calcium glycerophosphate now associated with only 1 molecule of water of hydration. Thus a change in constitution has occurred, and the more soluble but unstable salt with 2 molecules of water has reverted to the less soluble but stable salt with 1 molecule of water.

These 2 α-hydrates were rendered anhydrous: the $2H_2O$ salt lost all the water at 140°C., but the $1H_2O$ salt had to be dried at 150°C. On exposing the anhydrous salts to the atmosphere, both absorbed moisture and returned to their original states of hydration, that is to say, to the $2H_2O$ and $1H_2O$ hydrates, and dissolved 1 in 25 and 1 in 100 of water respectively, just as they did before subjection to dehydration. Thus there are 2 hydrates chemically the same, but having different solubilities. Possibly, the structures of the two hydrates differ, and a different arrangement of the calcium and glycerophosphate ions and of the molecules of water in the lattices could explain the effects noted.

X-ray diffraction data of the two hydrates and of the corresponding dehydrated salts are given below.

TABLE V

X-RAY DIFFRACTION DATA OF THE TWO HYDRATES OF α-CALCIUM GLYCEROPHOSPHATE.
INTERPLANAR SPACINGS IN Å WITH APPROXIMATE RELATIVE INTENSITIES

Monohydrate	Dehydrated Monohydrate	Dihydrate	Dehydrated Dihydrate
13·0 vs.	14·8 vvs.	14·2 vs.	10·9 vvs. broad line
6·7 w.	about 4·0 s. diffuse band	7·1 mw.	4·19 mw.
about 4·0 w. diffuse band			
5·5 mw.	2·73 w.	5·17 m.	3·86 ms.
4·64 s.	1·99 w.	3·49 m.	3·48 ms.
3·34 mw.		2·22 vw.	3·27 w.
2·89 m.		2·05 vw.	3·04 mw.
2·75 w.		1·81 w.	2·57 w. broad
2·33 w.		1·72 w.	2·11 w. broad
2·10 vw.			1·85 vw.
1·83 w.			1·73 vw.

s Strong.   m Medium.   w Weak.

These experimental data demonstrate the distinct structural difference of the two hydrates and of their respective dehydrated salts. It is interesting to compare the effect of dehydration on the diffraction patterns, and in particular on the strong high spacing lines, in the 2 cases. For the dehydrated monohydrate the pattern is almost non-existent except for a strong diffuse band and an extremely strong line cf higher spacing than the maximum spacing line of the original monohydrate. This suggests that the water molecules are important in holding the calcium glycerophosphate units of the structure together, and that on their removal these units drift apart and the structure becomes much less ordered. On the other hand, the dehydrated dihydrate has a good strong pattern with a decrease in spacing of the maximum spacing line from that of the original dihydrate. This suggests that on removal of the water mole-

cules the remaining calcium glycerophosphate units can pack together in a highly ordered crystal structure which can, however, readily revert to that of the original hydrate.

COMMERCIAL SAMPLES OF CALCIUM GLYCEROPHOSPHATE

Samples of this salt were obtained from the principal manufacturers and subjected to the B.P.C. tests: the results are given in Table IV. The last three samples in the table were prepared by the present authors.

TABLE VI

ANALYTICAL DATA OF SAMPLES OF CALCIUM GLYCEROPHOSPHATE

Sample	Loss at 150°C.	Assay by titration	Assay by ignition	α-isomer	Solution 1 in 50
	per cent.	per cent.	per cent.	per cent.	
A	7·6	93·0	99·46	79·76	Soluble ; but flocculent. Precipitate overnight.
B	10·8	94·60	98·80	78·92	Soluble but within 1 hour commenced to flocculate.
C	7·5	94·18	101·16	79·64	Not complete ; also commenced to flocculate on standing.
D	10·5	93·90	97·85	51·91	Soluble ; flocculated on standing.
α-salt 2H₂O	13·9	98·90	99·63	99·15	Soluble ; only very slight flocculation overnight.
α-salt 1H₂O	7·0	99·33	99·5	100·90	Not complete but dissolves quickly 1 in 120 to a clear solution.
β-salt 1H₂O	9·8	100·22	100·0	absent	Not complete but dissolves slowly 1 in 120.
B.P.C. Standards	Not more than 15·0	94·50	98·0	—	Dissolves with slight turbidity.

All figures are based on material dried at 150°C. ; B.P.C. directs drying at 130°C.

Reference to Table VI will show that 3 out of the 4 samples analysed failed to meet the standard set for titratable matter, and yet reached the requirement (or practically so) as regards ash. This at once brings up the question as to whether the preparations or the standards are wrong; and when the different solubilities are also taken into account, emphasis is added to the question. It is clear that there are some difficulties in manufacture, for otherwise there would be greater uniformity in the products, and it may be asked whether the requirements of medicine are such that more precise standards, to secure a closer approach to the pure salt, should be set up or whether such differentiation is of little therapeutic importance and wider limits should be allowed: such wider limits being, of course. rather arbitrary and merely arranged to suit good commercial practice. But on the question of solubility there is room for debate: insisting upon a degree of solubility which is not a stable property of the salt serves no useful purpose, but rather the reverse. If a pharmacist looks to a salt being soluble 1 in 50 of water and then, when using it in compounding, finds precipitation takes place, he is perturbed and at a loss: but if he knows that an ultimately stable solution can only exist at 1 in 100 some of his difficulties will not arise. Again, if there is no sound therapeutic reason for preferring one or other of the isomers, then degree of solubility in water is of still less importance since both salts will be readily soluble in the acid gastric juice.

PROPOSED CHANGES TO THE B.P.C. MONOGRAPHS FOR SODIUM AND CALCIUM GLYCEROPHOSPHATE

Resulting from their present investigation the authors suggest that the following changes might be made in the B.P.C. monographs.

Sodium Glycerophosphate

1. To omit the chemical formula and also the description referring to the material as " the sodium salt of β-glycerophosphoric acid." Instead, the description to be " Sodium glycerophosphate may be the sodium salt of α-glycerophosphoric acid $C_3H_7O_6PNa_2,6H_2O$ or the sodium salt of β-glycerophosphoric acid $C_3H_7O_6PNa_2,5H_2O$ or any mixture of these isomers."

2. The assay to remain as at present, but referred to the material dried at 150°C. Standards to be altered to " Not less than 98 per cent. and not more than 102 per cent. of $C_3H_7O_6PNa_2$ calculated on the material dried at 150°C."

3. Loss at 150°C. not to exceed 34·5 per cent. This admits the hexahydrate with 33·34 per cent. of water and allows a little additional moisture.

Sodium Glycerophosphate 50 per cent. Liquor

1. To be described as " An aqueous solution containing about 50 per cent. w/w of α-sodium glycerophosphate hexahydrate, or a mixture of the α and β-isomers."

2. The assay to refer to the equivalent of anhydrous sodium glycerophosphate in the liquor. The limits to be 32·6 per cent. and 36·0 per cent. of $C_3H_7O_6PNa_2$.

These limiting figures are derived from the following considerations —50 per cent. of the hexahydrate is represented by 33·6 per cent. of anhydrous salt, which becomes 32·6 per cent. when allowing for a purity limit of 98 per cent. Similarly 50 per cent. of the pentahydrate is equivalent to 35·3 per cent. of anhydrous salt, and this, calculated to the upper limit of 102 per cent. allowed for the crystalline salt, becomes 36·0 per cent.

3. The specific gravity range to be from 1·255 to 1·300. This covers the two limits of the assay and also allows for the presence of 2 per cent. of glycerin.

All the other present limits, including that for glycerin, could remain since no difficulty has been experienced in meeting these standards.

Calcium Glycerophosphate (Dihydrate) ... Now included in the B.P.C.

1. The loss on drying to be determined at 150°C.

2. The assay should be the method of Bennett and Campbell[10] in which igniting with ammonium nitrate and reigniting with nitric acid is adopted. The present authors found this method very satisfactory.

3. 1 g. of the salt should dissolve in 50 ml. of water at a temperature below 20°C. within a few minutes. On further dilution to 100 ml. and standing overnight no more than a very slight precipitate should develop.

The test for titratable matter and other limiting tests allow ample margin for the manufacturers' difficulties in production.

Calcium Glycerophosphate (Monohydrate) . . . Not recognised in the B.P.C.

1. It is recommended that official recognition be given to both α and β-salts both approximating to the monohydrates.

These are the salts which are stable in water, and which satisfy all the official requirements at present set out for the dihydrate with the single exception of solubility; the present requirement being that of an unstable condition.

1 g. of the salt should dissolve in 130 ml. of water within a few minutes. Both salts will dissolve in a smaller quantity of water, but the rate of dissolution is slow with the β-salt.

SUMMARY

1. A method has been described for the determination of the α-isomer of glycerophosphoric acid.

2. The preparation and properties of pure crystalline α- and β-sodium glycerophosphate have been described.

3. The isolation of "an impurity," occurring from a side reaction in the primary combination and which seems to approximate to a diester, is reported.

4. Two types of α-calcium glycerophosphate have been prepared: a dihydrate which is unstable in water and a monohydrate which is stable.

5. The properties of the two hydrates of the calcium salt have been investigated and differences in their physical behaviour are reported.

6. The preparation and properties of β-calcium glycerophosphate have been described.

7. Commercial samples of calcium glycerophosphate have been examined and their departure from official standards noted.

8. Suggestions for the official monographs have been made.

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DISCUSSION

The paper was presented by Mr. J. S. Toal.

Mr. G. J. W. Ferrey (Manchester) said that 23 years ago it was shown that the calcium glycerophosphate then on the market was the monohydrate, and not the dihydrate which was introduced into the Codex later. The 50 per cent. solution of sodium glycerophosphate was standardised by some makers on anhydrous sodium glycerophosphate and by others on the basis of the salt containing $5\frac{1}{2}$ molecules of water of crystallisation, while others varied between those two limits. There was very little diester in some makes. The insolubility of calcium glycerophosphate had been a difficulty in making the compound syrup. He wondered whether it would not be best to leave out the calcium glycerophosphate altogether and put in some soluble form of phosphoric acid; the main object of putting glycerophosphate in was to get the tonic effect of the phosphorus, and they could very well dispense with the calcium. He asked whether there was any difference in therapeutic activity between the α-and β-glycerophosphoric acids, and also whether there was any evidence to show that glycerophosphoric acid and its salts had any therapeutic action which was not possessed by phosphoric acid and its salts.

Dr. G. E. Foster (Dartford) asked for information about magnesium glycerophosphate. This formerly came from Germany and usually complied with the requirements of the British Pharmaceutical Codex. After the war, however, it had been difficult to get any of British manufacture which complied.

Dr. K. Bullock (Manchester) criticised the author's statement that the solubility of one of the isomers was not of great importance, because in any case it would dissolve in the acid gastric juice. Surely the salt would be reprecipitated again in the intestine; few substances were absorbed from the stomach. If, on the other hand, it was a real decomposition, why should not the separate products be given, instead of combining them and then allowing them to disintegrate again in the stomach?

Mr. R. W. Gillham (Leeds) asked whether the authors considered that their work had thrown any light on the problem of the precipitation which occurred in samples of compound syrup of glycerophosphates.

Mr. J. S. Toal, in reply, referring to the calcium salt, said that one could get the dihydrate only by precipitation with alcohol. The two hydrates had different solubilities. The dihydrate would dissolve with a 1 in 20 initial solubility, and a stable solubility of 1 in 100. The monohydrate had an initial solubility of 1 in 100. They had found that 50 per cent. solutions were low in diester. He suggested that the formula for the compound syrup of glycerophosphates ought to be based on the pure salts; as it was now each manufacturer had a different preparation, and they did not comply with the B.P.C. With one or two exceptions, none of the magnesium salts on the market to-day would pass the Codex tests. The standard required 97 per cent. by ignition and 95 per cent. by titration, but many of them were less than 90 per cent.

BACTERIAL SURVIVAL IN SYSTEMS OF LOW MOISTURE CONTENT

PART I. ENUMERATION AND DISTRIBUTION OF ORGANISMS IN SPRAY-DRIED POWDERS

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INTRODUCTION

THE growth and metabolism of bacteria and their response to altered environmental conditions when growing in liquid media, or such media solidified by addition of a small percentage of agar, have been extensively studied both quantitatively and qualitatively. Much has been written about the enumeration of bacteria suspended in liquids. On the contrary, the behaviour and enumeration of bacteria in powders, pastes, and oils, that is to say in systems of relatively low moisture content, have been studied to a much lesser extent. This situation is probably due to the difficulty, experienced in the past, of obtaining powders of predetermined composition, containing an even distribution of suitable numbers of a known organism, with which the experiments could be carried out.

As long ago as 1909 Shackell[1] called attention to the possibilities and advantages of freeze-drying for the purpose of obtaining bacterial cultures in a form in which they would retain their viability, cultural characteristics and state of virulence. Swift[2], and Elser, Thomas and Steffan[3], showed that hæmolytic streptococci and meningococci were resistant to freeze-drying and subsequently remained viable over a long period of time. Heller[4] used this method of drying for the quantitative investigation of environmental factors affecting dried samples of *Streptococcus pyogenes* and *Escherichia coli*. He did not, however, specify his limits of error. The products of freeze-drying tend to occur as flakes, and an examination of the literature has not revealed any report establishing that viable micro-organisms are uniformly distributed in the powders formed from such products.

The work described in the present communication was foreshadowed in a previous paper from this Department[5]. It was there suggested that a spray-dried powder containing a known species of micro-organism in a medium of known composition might prove suitable for the study of the effects of environmental conditions on the organism in such a powder, or such a powder suspended in oil.

Before enquiry into the effects of environmental conditions can be made, certain facts must be established. Organisms suitable for the investigations on hand must be chosen. It must be shown that these organisms can be counted satisfactorily, or at any rate that reasonably concordant replicate viable counts can be obtained and the errors of such counts must be statistically evaluated. It must be shown that the viable organisms counted are uniformly distributed in the powder or that with suitable treatment such uniformity can be attained. The organisms used should remain viable for considerable lengths of time in the powder,

but if they cease to be able to reproduce during the course of the experiment then it must be possible to estimate the extent of such degeneration over the periods of time involved in the experiment. It should clearly be understood that the fact that a particular bacterium does not produce a colony when the medium is plated out may not mean that the organism is dead as an individual, but it does mean that it is incapable of reproduction. In the following work such organisms will, as is usual, be regarded as non-viable.

EXPERIMENTAL TECHNIQUES

Choice of Test Organisms. Considerable thought has been given to the question of the choice of suitable organisms. For the early work on spray-drying it was essential that the test organisms should be non-pathogenic since some of the dried culture might be inhaled. Preliminary experiments with yeasts and moulds did not appear promising, largely on account of difficulties of culture and enumeration. Bacteria, although smaller in size and so more difficult to examine microscopically and distinguish from unorganised matter in total counts, yielded more repeatable figures.

It was considered that both spores and vegetative forms should be examined in this work, for the following reasons. (i) To be satisfactory, suggested processes for sterilisation must destroy the viability of spores, but (ii) a substance which will kill, or even profoundly inhibit vegetative organisms, even if it does not affect spores, may be considered to be satisfactory for the maintenance of sterility in, for example, multiple dose containers. (iii) For the understanding of the life processes of bacteria the behaviour of both spores and vegetative forms is of interest.

Bacillus subtilis appeared to be the most obviously satisfactory non-pathogenic sporing organism. To perform " useful " viable counts on *B. subtilis* in the vegetative form is probably an impossibility owing to the tendency of the cells to form chains and the tendency of these chains to form a matted growth or pellicle on the surface of liquid media. It is difficult, not only to break down the chains into individual cells, but the pellicle is very difficult to wet so that it has proved impossible to obtain the even suspension of organisms necessary for concordant replicate counts. When, however, *B. subtilis* is allowed to spore on the surface of agar each spore is formed individually in a separate bacterial cell. This process is followed by the autolysis of the original vegetative cells; the whole surface becomes moist and the spores can easily be mixed with a suspending fluid. Microscopical examination of the resultant suspension shows the spores to be separate with no tendency to clump. Such a suspension has proved very satisfactory in this work. The details of preparation are as follows.

The organism used was *Bacillus subtilis* (Marburg, No. 3610) obtained from the National Collection of Type Cultures. The surface growth on 10-day agar slopes was washed off with 20 ml. of sterile water, and the resulting suspension was suitably diluted and distributed in glass

ampoules. These were heated at 80°C. for 3 minutes, to destroy any vegetative organisms, and were then stored in a refrigerator. Such suspensions showed no significant decrease in viable count after 6 months' storage.

To find a suitable non-sporing organism has proved to be much more difficult. *Bacterium lactis aerogenes,* which was used in the work described in the previous paper, is non-pathogenic, evidences no marked tendency to chain formation and gives rise to colonies which can easily be counted, but over 99 per cent. of the bacteria are usually destroyed on spray-drying and the survivors fairly rapidly die off in the resultant powder under ordinary conditions of storage. It is possible that some of the difficulties are inherent in the nature of vegetative bacteria. Recently it has been found that *Streptococcus fæcalis* appears to be more resistant to drying and storage in the resultant powder, and provided that difficulties involved in obtaining a viable count of a *Streptococcus* can be overcome, this organism may prove very satisfactory. For the present, however, the use of *Bact. lactis aerogenes* has been continued, the strain used being No. 418, obtained from the National Collection of Type Cultures. The details of its use are as follows. For each spray-drying 10 ml. of peptone water was inoculated with the organism and incubated at 30°C. for 24 hours. The suspension so obtained was thoroughly mixed by means of a sterile pipette and added to the solution to be dried.

Choice of Method for Viable Counts. Wilson[6] advocated a method for counting viable organisms, using roll-tubes which were inoculated by means of dropping pipettes, and the accuracy of the technique has been established by Withell[7] and others. Anderson and Stuart[8] and Miles and Misra[9] used dropping pipettes to perform " surface-viable " counts. By this method surface growths of *B. subtilis* tend to spread, and since only a relatively small number of colonies are counted errors due to interference from spreading are magnified by the large multiplication factor involved. To minimise the difficulty Davis[10], using this method, reduced the colony size by incorporating sodium taurocholate in the medium, but, while he obtained a satisfactory statistical uniformity, he found the salt to have an inhibitory effect. This might operate unequally in the presence of other chemical agents. Such inhibition would be detrimental to the objects of the present investigation. With dropping pipettes as used by the above workers, the size of drop delivered is not only governed by the external diameter of the tip, but is also influenced by the temperature and viscosity of the liquid dropped. They are not, therefore, particularly suitable for measuring samples of liquids where the viscosity may differ, as is the case with original and reconstituted samples before and after drying. Snyder[11] compared the use of dropping pipettes with that of graduated pipettes in combination with both roll-tubes and plates and with surface counts. He found graduated pipettes to be more accurate, but the significance of the difference was lost when estimating viable counts because the pipetting

errors contributed only a small proportion of the total error. It was decided, therefore, to use pipettes marked with two graduations for the delivery of 1 ml. for dilutions and inoculations: roll-tubes were used for the counts, which were carried out in the following manner. 9 ml. of diluent were placed in each of a number of plugged test-tubes. 1 ml. of the suspension to be counted was pipetted into the first of these tubes. A second pipette was used to mix the contents of the tube and to transfer 1 ml. of the mixed suspension to the second tube. A suitable number of such serial dilutions was made, a fresh pipette being used for each transference. 1 ml. portions of the final dilution were inoculated into roll-tubes containing 5 ml. of melted agar medium which had previously been maintained at a temperature of 46°C. The roll-tubes, after inoculation, were held horizontally under a stream of cold water and rotated until the agar set. They were then placed, plug-downward in the incubator. The accuracy of the technique has been assessed statistically according to the method used by Withell[7], Davis[10], Berry and Michaels[12] and others. In the immediately following paragraphs the results refer to experiments with *B. subtilis* spores. A summary of the corresponding results with *Bact. lactis aerogenes* is given on pages 891 *et seq.*

The Accuracy of the Graduated Pipettes. The pipettes used were made of fine bore glass tubing tapered at one end to a stout point. A ring was marked round the tube about 2 cm. from this end, and a second mark made at a distance above this corresponding to a delivery of 1 ml. The pipettes were calibrated gravimetrically. The weights of water delivered by the pipettes were all greater than 0·9873 and less than 1·0073 g. and the mean deviation from the theoretical values (0·9973 g.) was 0·0043 g., giving a mean percentage deviation of 0·431 per cent. Jennison, Marshall and Wadsworth[13] stated that pipettes suitable for viable counts should have an accuracy within ± 1 per cent.

In a viable count, however, the delivery of liquid is not controlled as carefully as in the above calibration. Twenty pipettes were taken at random and fitted with rubber teats by means of which water could be drawn up to the upper mark and ejected until the meniscus reached the lower mark. The water so discharged was weighed, and the process repeated 3 times for each pipette. In order to make the conditions as severe as possible no specific time was allowed for drainage, and the various pipettes when being filled were inserted at different depths into the water. The results are given in Table I.

From the weights of water discharged from the same pipette the variance was calculated and from the mean of these the co-efficient of variation was found to be 0·77 per cent. From the same figures three variances of the weights of water discharged from different pipettes were also calculated and from the mean of these the co-efficient of variation was found to be 1·32 per cent. These figures compare with 1·03 per cent. and 1·83 per cent. obtained by Withell[7], using dropping pipettes.

C

The Accuracy of the Dilution Technique. In most " viable counts," 10^{-1}, 10^{-2}, and 10^{-3}, dilutions have been prepared, the last dilution being tubed. Each dilution was prepared by pipetting 1 ml. of suspension into 9 ml. of diluent. As diluting agent quarter-strength Ringer's solution was used with *Bact. lactis aerogenes* while distilled water was used with *B. subtilis* since it was found to have no destructive effect on spores.

TABLE I

ERRORS INVOLVED IN MEASURING 1 ML. OF WATER WITH PIPETTES, FILLING AND EMPTYING BEING EFFECTED BY A RUBBER TEAT

Weights			Mean ($\bar{x}$)	$S(x-\bar{x})^2$	Variance
0·9830	0·9825	0·9800	0·9818	0·00000517	0·000002585
0·9950	0·9920	0·9750	0·9873	0·00023267	0·0001 6335
0·9793	0·9687	0·9620	0·9700	0·00015218	0·0000 6090
0·9730	0·9809	0·9651	0·9730	0·00012482	0·000062410
0·9945	1·0055	0·9972	0·9991	0·00006573	0·000032865
0·9865	0·9700	0·9820	0·9795	0·00014550	0·0000 2750
0·9785	0·9560	0·9793	0·9713	0·00034993	0·000174965
0·9845	0·9822	0·9864	0·9843	0·00000886	0·000004430
0·9785	0·9934	0·9841	0·9853	0·00011329	0·000056645
0·9718	0·9867	0·9753	0·9779	0·00012141	0·000060705
0·9994	0·9831	0·9834	0·9886	0·00017393	0·000086965
1·0000	1·0100	0·9916	1·0005	0·00016971	0·000084855
1·0020	1·0032	0·9928	0·9993	0·00006475	0·000032375
0·9792	0·9960	0·9920	0·9891	0·00015403	0·000077015
1·0086	0·9925	0·9945	0·9985	0·00015401	0·0000 7005
0·9731	0·9807	0·9910	0·9816	0·00016142	0·000080710
1·0050	1·0058	1·0000	1·0036	0·00001976	0·000009880
0·9873	0·9841	0·9959	0·9891	0·00007448	0·0000 7240
0·9807	0·9800	0·9759	0·9789	0·00001345	0·000006725
1·0069	1·0131	1·0117	1·0106	0·00002115	0·000010575

Mean ($\bar{x}$) ...	0·9883,	0·9888,	0·9858	Total	0·0011 63125
$S(x-\bar{x})^2$ ...	0·00272404,	0·00419382,	0·00274324	Mean Variance ...	0·000058156
Variance ...	0·00014337,	0·00022073,	0·00014438	S. Deviation... ...	0·007626

M. Variance	0·00016949	Co-efficient of
S. Deviation	0·01302	Variation ... ... 0·77%
Co-efficient of Variation ...	1·32%	

Wilson[14] showed that distilled water was lethal to vegetative organisms and found quarter-strength Ringer's solution to be satisfactory for suspending such an organism. He also showed that distilled water had a dispersive effect on clumps of bacteria occurring in milk. The 9-ml. quantities of diluent were delivered into the test-tubes from a burette consisting of a graduated 10-ml. pipette, plugged at the upper end with cotton wool, and connected at the lower end by means of a two-way tap, either to a flask containing the diluent, or to a hooded nozzle from which the diluent could be measured into a test-tube. The co-efficient of variation of ten 9-ml. samples delivered from the apparatus was found to be 0·287 per cent.

In order to estimate the overall error of diluting and pipetting, 20 serial dilutions were carried out. From each of 20 spore suspensions, 10^{-1}, 10^{-2}, and 10^{-3} dilutions were prepared in duplicate. Five roll-tubes were then inoculated from each of the 10^{-3}, dilutions giving 2 sets of 5 tubes from each spore suspension. The results are given in Table II.

The variance of the mean counts obtained from each pair of dilutions was calculated and from these the mean co-efficient of variation was found to be 3·06 per cent. Another similar experiment by a different

worker gave a mean co-efficient of 3·37 per cent. These figures may be compared with 4·95 per cent. obtained by Withell[7] and 3·79 per cent. obtained by Berry and Michaels[12].

The Error of Counting Colonies of B. subtilis. If a roll-tube be counted on successive occasions counts will be obtained which differ slightly from one another. The differences may be attributed to:—(a) Inability to recognise small colonies; (b) The appearance of "double" colonies where two colonies lie one above the other in the medium; (c)

TABLE II

ERRORS OF DILUTING AND PIPETTING

Experiment	1st 10⁻³ dilution (mean count of 5 tubes)	2nd 10⁻³ dilution (mean count of 5 tubes)	Variance
1	168	163	12·5
2	218	219	0·5
3	199	197	2·0
4	186	195	40·5
5	187	195	32·0
6	206	203	4·5
7	208	201	24·5
8	212	219	24·5
9	196	204	32·0
10	212	226	98·0
11	237	253	128·0
12	131	124	24·5
13	119	128	40·5
14	124	120	8·0
15	159	160	0·5
16	134	131	4·5
17	132	137	12·5
18	135	129	18·0
19	78	72	18·0
20	79	75	8·0

The suppression of some colonies by local crowding; (d) The occurrence of spreading surface colonies both at the medium-air interface and the medium-glass interface; (e) The development of daughter colonies arising from the surface colonies; (f) Parallax error. Colonies which have been counted are identified by marks made on the surface of the glass. As the tubes are rotated, the relative positions of the marks and of the colonies lying deeper in the agar may alter so that colonies which have been marked appear to be unmarked and vice versa.

Wilson[14] investigated the first three of these sources of error. He concluded that the use of a small hand lens magnifying 2 or 3 diameters greatly aided the recognition of small colonies. He also examined the structure of double colonies appearing as circular surface colonies upon which smaller, lenticular colonies, lying deeper in the agar, were superimposed, and suggested that where the lenticular colony was placed centrally with respect to the circular colony these should be regarded as one, whilst when the lenticular colony was eccentrically placed the two colonies should be regarded as separate. Wilson also showed that if the tubes are overcrowded suppression of some colonies may occur and recommended that the count should lie between 30 and 300. All these recommendations have been followed in the work described in this paper.

The presence of spreading surface growth has been generally held to

cause low values for the viable count and various attempts have been made to reduce the tendency of such colonies to form and to reduce their size when they do occur. Thornton[15] endeavoured to control them by reducing the nutrient content of the agar medium but the period of incubation had to be extended to 10 days which is unsuitable for roll-tubes because they dry out unless the incubator atmosphere is kept saturated with water, in which case the surface growth is greatly increased. Various methods of drying the surface of the agar before incubation were tried but all proved unsatisfactory.

The surface growth may affect the count in two ways. Firstly, it may become confluent with other colonies growing on the surface. The proportion of these, however, is small and the surface growth occupies usually only one-third or less of the total surface. The number of colonies affected in relation to the total number occurring in the tube is therefore not likely to be large. Moreover, discrete colonies are often observed lying on the surface in the middle of the spreading growth and surrounded by a clear ring, which suggests that during the period of incubation they have not lost their identity. Secondly a more serious difficulty is caused by the presence at the edge of the spreading growth of numbers of small discrete daughter colonies, produced on the surface from it. These colonies differ in appearance from those submerged colonies normally seen in tubes where no spreading growth occurs; differentiation was based on the following characteristics.

Submerged Colonies are small, irregular, or "woolly" colonies, $\frac{1}{2}$ to 1 mm. in diameter, opaque, white or pale cream by reflected light, brown by transmitted light.

Surface Colonies may be produced from the submerged colonies. When these have grown to reach the upper surface they produce a spreading colony thereon. The size of this depends on the amount of moisture present at the surface of the medium. During incubation this moisture film gradually dries up, so that the depth at which an organism is implanted in the medium and the speed with which it grows govern the size of surface colony produced. Thus many colonies produce no surface growth. Others produce circular surface colonies; these are thin, or slightly raised, the surface being smooth or showing radiate ridges or wrinkles; the submerged colony can be seen as a denser spot lying below the centre of the circular colony. More advanced surface colonies show transitions from the circular type through lobate or pinnatifid forms to much-branched, somewhat radiate, moderately thin, colonies, the ends of the branches and their subdivisions being club-shaped. A small submerged colony can be seen at the centre of radiation. In the largest " spreaders " the centre is occupied by a continuous film of growth. Since these have probably been produced from organisms implanted on or very near the surface of the medium there is no submerged colony apparent. Surface colonies are occasionally produced at the medium-glass interface. These are very thin with irregular edges and are of an even, granular texture.

Daughter Colonies are sometimes produced from the edges of extensive

surface " spreaders." They are circular or scaly, effuse and much thinner than the " spreader " or the circular colonies described above. Since they are not produced from submerged colonies they show no central spot. They occur in groups the members of which are similar in size and texture and the presence of submerged colonies growing below them can usually be easily recognised. Daughter colonies may also be formed from colonies at the medium-glass interface. They resemble their parent colonies but occur in well-marked groups.

It appeared that it should be possible to perform a " viable count " satisfactorily even in the presence of the surface growths. Counting tests were therefore performed to determine the effect of these growths and of the parallax error.

Twenty tubes were taken and each was counted three times. Three methods of performing the count were used, as follows: —

Method (1). 8 longitudinal lines and 3 transverse rings were marked on each tube with a wax pencil. The tube was thus divided into small areas within which the colonies were counted. Colonies touching the line were counted only on the upper and right hand margins of each area. All colonies including indistinct and daughter colonies were counted.

Method (2). As above, but any daughter colonies or colonies rendered indistinct by the surface growth were ignored. A new set of tubes was used, half the tubes having a high count and half a lower count.

Method (3). As in (2) but marking the tubes with 1 longitudinal line and 6 transverse rings.

The results are set out in Table III.

TABLE III

Method (1		Method (2)		Method (3)	
Counts	Variance	Counts	Variance	Counts	Variance
347 350 346	4·5	436 431 432	7·0	433 426 430	12·5
610 599 617	81·5	463 465 475	41·5	463 453 465	41·5
325 324 322	3·5	404 389 419	226·5	409 407 409	1·5
563 566 561	6·5	448 422 437	170·5	393 394 398	7·0
546 549 527	142·5	408 384 394	145·0	443 459 443	85·5
598 590 596	17·5	464 448 453	65·0	400 400 408	21·5
537 522 486	687·0	420 385 399	310·5	408 395 394	61·0
400 402 398	4·0	431 395 402	364·5	394 399 401	13·0
457 434 432	176·5	421 393 391	281·5	431 429 432	2·5
369 364 374	25·0	194 189 191	6·5	193 194 193	0·5
344 356 356	48·0	167 170 165	6·5	172 173 171	1·0
339 334 331	18·0	196 197 201	7·0	189 199 192	26·5
339 322 334	76·5	186 192 188	9·5	197 187 182	58·5
273 275 290	86·5	159 160 158	1·0	177 160 161	91·0
305 277 278	253·0	202 198 191	31·0	215 225 223	28·0
277 265 273	37·5	193 196 188	16·5	194 188 188	12·0
249 282 287	426·5	177 177 181	5·5	178 178 178	0·0
281 271 276	25·0	186 184 189	16·5	193 184 186	22·5
272 257 272	75·0	206 209 207	2·5	196 201 195	11·5
275 273 277	4·0	211 210 209	1·0	205 214 210	20·5
	2198·5		1708·0		518·0

Overall variance ...	=109·9	Overall variance	= 85·4	Overall variance	= 25·9
Standard deviation	=10·48	Standard deviation	= 9·24	Standard deviation	= 5·09
S.E. of mean of three counts	=6·098	S.E. of mean of three counts ...	= 5·34	S.E. of mean of three counts ...	= 2·94

The Standard Error of the Mean of Three Counts using Method (1) is 6·098, and the majority of the variances are excessive. The effect of the surface growth, either by virtue of obliteration of other colonies or by masking of them at its fringes, would be greatest on the lower counts. The use of Method (2) should minimise the effect. On the other ha.nd, the parallax error should not be considerable on tubes of low count, for the distance separating the colonies is greater and the number of colonies bordering the demarcation lines fewer than in tubes of high count. The experimental results of Method (2) are in agreement with this, the variances of the lower counts being significantly improved, while those of the higher counts show no such improvement. Using Method (3) the parallax error has been minimised, for this occurs almost entirely along the longitudinal markings, since a slight rotation of the tube easily brings a colony from one side of the line to the other. Using this method the variances of both high and low counts were satisfactory and the Standard Error of the Mean of Three Counts was found to be 2·94, comparing favourably with that of 2·44 obtained by Berry and Michaels[12] using *E. coli.* It may be mentioned that a different worker using Method (2) obtained a Standard Error of 4·73. The same worker using Petri dishes instead of roll-tubes obtained the figure of 7·26 for the Standard Error.

A direct estimate of the effect of the surface growth on the viable count was also afforded by a series of tubes inoculated from the same suspension, in which a considerable variation occurred in the size of surface colonies present. The results are given in Table IV.

TABLE IV

THE EFFECT OF SURFACE GROWTH ON THE VIABLE COUNT OF *B. SUBTILIS*

Count	Area of Surface Growth	Count	Area of Surface Growth
146	Extensive	143	Very slightly
137	Nil	111	Slight
133	Moderate	143	Nil
123	Slight	146	Nil

The highest count was obtained in two tubes, one of which bore an extensive surface growth, while the other showed none at all, and the tube with the lowest count had only a small " spreader." There is, in fact, no significant difference between the counts which can be attributed to the effect of the surface growth, and it appears that viable counts may be satisfactorily performed even in the presence of extensive spreading colonies.

The Suitability of the Agar Medium. As pointed out above, an organism is regarded as viable if it produces a colony in the roll-tube, and as non-viable if it fails to produce such a colony. The composition of the medium used in the roll-tubes is to some extent responsible for the rate of growth of the colonies and the ultimate size which they attain. It is even more important that certain, possibly damaged or weakened, individual cells may fail to grow in one medium, while they produce a

colony and so give rise to a higher count in a more favourable substrate. The medium is, therefore, very important. For work such as that described in this paper it should (i) be accurately reproducible, (ii) be uniform throughout, all the ingredients preferably having been in solution, (iii) give concordant replicate counts from time to time and from batch to batch, (iv) give maximal counts for any given bacterial suspension.

Broadly speaking, three types of media are available: (i) Synthetic media prepared entirely from pure chemicals. Different batches may be identical, but they are difficult to prepare and opinion is by no means fixed as to the proportions of ingredients which they ought to contain. Their sensitivity is likely to vary greatly with the addition or omission of trace substances or vitamins. (ii) At the other extreme are the media prepared by direct extraction of meat tissues, with or without addition of serum. Such media are very sensitive, often giving the highest counts, but there is no guarantee of their constancy of composition from batch to batch. (iii) In between the above classes are the media prepared from peptone, with or without addition of commercial meat extracts. These have the advantages of being moderately sensitive and yet reproducible, for relatively large samples of peptone and meat extract can be purchased so that the batches of media prepared from them during the course of a lengthy set of experiments will not vary detectably. For these reasons this type of medium was chosen and three examples were examined. They consisted of:—

A. 3 per cent. of agar with 2·0 per cent. of peptone and 0·5 per cent. of sodium chloride.

B. The same with the addition of 0·5 per cent. of proteolysed liver extract.

C. The same as A with the addition of 0·5 per cent. of Lab-Lemco.

To prepare the media the ingredients were dissolved in distilled water, adjusted to pH 7·6 with N caustic soda, solution being effected by heating in an autoclave at 10 lb. pressure. The solution was filtered through washed sand and filter-paper pulp. The medium was then sterilised by autoclaving at 10 lb. pressure. The final pH was 7·2.

To compare the media, 25 roll-tubes of each were inoculated with 1 ml. from the same suspension of the test organism. Each of these batches was divided into 5 groups of 5 tubes which were incubated at different temperatures, the same 5 temperatures being applied to all three batches. The results are given in Table V.

The mean counts obtained with medium C are lower than those obtained with the other media. The counts on Medium A at temperatures between 26°C. and 42°C. do not differ significantly from those on Medium B, but the surface growth was thicker on the latter. Medium A was, therefore, chosen for use with B. subtilis.

Thornton[15] suggested that batches of media should comply with two requirements: (i) Different batches should give reproducible results. (ii) Parallel platings from the same batch should develop the same number of colonies within the limits of sampling variance.

TABLE V

THE EFFECT OF TEMPERATURE AND COMPOSITION OF MEDIUM ON THE VIABLE COUNT OF *B. SUBTILIS*

	Medium A					Medium B					Medium C				
	Incubation Temperature					Incubation Temperature					Incubation Temperature				
	20°C	26°C	32°C	37°C	42°C	20°C	26°C	32°C	37°C	42°C	20°C	26°C	32°C	37°C	42°C
	90	131	155	127	120	79	112	136	198	75	53	72	77	82	121
	109	136	135	133	122	36	110	126	139	94	56	95	88	89	126
	86	118	120	120	127	7	131	116	107	115	57	73	77	83	138
	103	111	122	142	138	10	149	125	112	85	57	95	127	97	103
	86	141	145	106	108	22	124	137	124	97	44	80	114	93	117
Mean ...	95	127	135	126	123	17	125	128	116	93	53	83	97	89	121

To test requirement (i), 5 tubes were rolled from each, using the same suspension of the test organism in each case. The results for successive batches are given in Table VI.

TABLE VI

THE REPRODUCIBILITY OF VIABLE COUNTS ON SUCCESSIVE BATCHES OF MEDIA

Batch I					Mean	Batch II					Mean	T	P
Count						Count							
82	90	76	86		84	118	71	92	85		91	0·462	0·6 to 0·7
97	146	112	111	116	116	115	123	123	119	116	119	0·363	0·7 to 0·8
126	140	127	130	110	126	125	113	120	133	110	120	0·942	0·3 to 0·4

The probability is in each case satisfactory and it may be concluded that different batches of the medium can be prepared having the same sensitivity to the test organism.

Requirement (ii) can be tested by the use of the statistic x^2 calculated in the form

$$x^2 = \frac{S(x-\bar{x})^2}{\bar{x}}$$

Berry and Michaels[12] tested each batch of medium by counting 20 replicate tubes and comparing the value of x^2 obtained from them with that to be expected if the variation involved only the normal sampling variance. For comparison, results of similar tests are included here. Table VII shows a typical result for one batch and Table VIII shows the summarised results for all the batches used in the present experiments. In all cases P was found to be satisfactory.

This test, however, is really a test of all the errors involved in carrying out the count and while, assuming all other errors to be small, the agar may be assumed to be satisfactory, the test is not sufficiently comprehensive to assess the overall error. Moreover, such a test would conceivably

be of use in testing media used with mixed bacterial cultures where, for instance, the encouragement of certain fast-growing organisms might militate against the development of slower growing colonies, but it is difficult to see how a homogeneous medium could increase the variance of counts on a pure culture.

The Duration and Temperature of Incubation. The optimum temperature for growth of *B. subtilis* is given by Bergey[16] as 30° to 37°C. and by Topley and Wilson[17] as 37°C. It is possible also that it may vary to some extent with the medium employed and a test was therefore carried out to examine this. Table V, to which reference has already been made, shows the counts resulting from incubation at various temperatures and on various media. In these experiments the temperature for maximal counts for *B. subtilis* appears to lie betwen 26°C. and 42°C. and incubation temperatures within this range were therefore used for this organism.

TABLE VII

GOODNESS OF FIT OF χ^2 OBTAINED FROM 20 REPLICATE ROLL-TUBES OF
B. SUBTILIS

Count (x)	Mean ($\bar{x}$)	$(x - \bar{x})$	$(x - \bar{x})^2$	$x^2 = \dfrac{S(x - \bar{x})^2}{\bar{x}}$
180		13	169	
181		12	144	
206		13	169	
192		1	1	
195		2	4	
180		13	169	
206		13	169	
181		12	144	
189		4	16	2782
207	193	14	196	——
203		10	100	193
183		10	100	= 14·42
180		13	169	
202		9	81	
211		18	324	
203		10	100	
190		3	9	
180		13	169	
178		15	225	
211		18	324	

N = 19 x^2 = 14·42   P = 0·8 — 0·7

TABLE VIII

SUMMARY OF VALUES OF χ^2 OBTAINED FROM SETS OF 20 ROLL-TUBES OF
B. SUBTILIS

Batch No.	No. of Tubes	N	X^2	P
1	20	19	14·42	0·8—0·7
2	20	19	17·98	0·7—0·5
3	20	19	14·13	0·8—0·7
4	20	19	13·17	0·9—0·8
5	20	19	15·60	0·7—0·5
6	20	19	18·59	0·5—0·3
7	20	19	11·83	0·9—0·8
8	20	19	14·81	0·8—0·7
9	19	18	11·83	0·9—0·8

In order to determine the incubation period for *B. subtilis* likely to produce the most reliable counts 5 tubes were incubated at 32°C. and counted at suitable intervals. The results are given in Table IX.

TABLE IX

EFFECT OF DURATION OF INCUBATION ON THE VIABLE COUNT OF *B. SUBTILIS*

Period of Incubation	Count	Mean	Variance
24 hours	160, 165, 143, 161, 115, 148	149	343
40 hours	168, 163, 145, 160, 150, 146	155	93
48 hours	171, 166, 146, 154, 150, 151	156	98
60 hours	175, 155, 146, 152, 148, 143	153	133

The mean counts show no significant differences but at 24 hours the variance of the counts appears excessive, probably because many of the colonies are too small to be seen satisfactorily. While the experiment is too small to permit of accurate conclusions, the mean count at 24 hours would appear to be unreliable. After 60 hours' incubation the surface growth is thicker and, while this has produced no significant diminution in count, it renders the process of counting more difficult. The most suitable incubation period for *B. subtilis* would therefore appear to be about 48 hours. The fact that the count has not diminished after 60 hours tends to confirm the findings discussed above that the surface growth has little effect upon the viable count.

Table X shows the result of another experiment in which temperature and duration of incubation were both varied. It suggests that a temperature between 32°C. and 40°C. should be used with an incubation period of about 48 hours.

TABLE X

EFFECT OF TEMPERATURE AND DURATION OF INCUBATION ON THE VIABLE COUNT of *B. SUBTILIS*

Period of Incubation	Temperature							
	26° C.		32° C.		37° C.		40° C.	
	Count	Mean	Count	Mean	Count	Mean	Count	Mean
24 hours ...	65, 58, 50, 55, 45	65	188, 192, 206, 220, 185	196	336, 246, 307, 245, 410	309	367, 414, 314, 429, 416	382
48 hours ...	89, 119, 105, 108, 111	106	222, 205, 223, 221, 242	223	442, 301, 369, 356, 420	374	401, 470, 345, 446, 405	393
120 hours ...	107, 154, 142, 130, 140	135	235, 226, 242, 217, 210	226	385, 330, 333, 346, 410	361	411, 378, 383, 392, 405	394
168 hours ...	105, 151, 141, 118, 131	129	240, 227, 235, 226, 221	230	390, 315, 326, 350, 411	357	397, 457, 383, 385, 407	386

The Normal Sampling Variance. Fisher, Thornton and Mackenzie[18] showed that plate counts approximated to small samples of a Poisson series and deduced from this that x^2, the Index of Dispersion, should exhibit a characteristic distribution which was tabulated by Elderton[19]. The distribution of the values of x^2 determined experimentally could then be compared with the hypothetical distribution, and the Goodness

of Fit determined. Such a comparison can be used to examine the overall errors of performing viable counts and provides a more critical test than the use of a single x^2. The values of x^2 obtained with 100 samples of spore suspensions of *Bacillus subtilis* have been tabulated in Table X and their Goodness of Fit tested.

The observed distribution of x^2 shows no significant departure from the theoretical distribution and the Probability obtained is satisfactory. It may be concluded, therefore, that the technique used is reliable and that accurate and reproducible results can be obtained with it.

Results of Test of Counting Technique using Bact. lactis aerogenes. Tables XI, XII, XIII, XIV and XV relating to *Bact. lactis aerogenes* correspond with Tables III, V, VII, IX and X respectively for *B. subtilis.* Table XVI shows that quarter-strength Ringer's solution is a suitable diluent for use with *Bact. lactis aerogenes.* These tables establish the fact that reliable and reproducible values for the viable count of this organism can be obtained using Medium A and incubating at 20°C. to 40°C. for 24 hours.

TABLE X

GOODNESS OF FIT OF VALUES OF x^2 OBTAINED FROM COUNTS ON SETS OF
FIVE ROLL-TUBES, USING *B. SUBTILIS*

Value of x^2			Expected Frequency (m)	Observed Frequency (m + x)	Difference (x)	$\dfrac{x^2}{m}$
Under 1	...	...	9·02	13	3·98	1·756
Between 1 and 2	...	...	17·40	11	−6·40	2·355
Between 2 and 3	...	...	17·79	19	1·21	0·082
Between 3 and 4	...	...	15·18	20	4·82	1·530
Between 4 and 5	...	...	11·87	9	−2·87	0·695
Between 5 and 6	...	...	8·82	8	−0·82	0·076
Between 6 and 7	...	...	6·33	6	−0·33	0·017
Between 7 and 9	...	...	7·48	8	0·52	0·036
Over 9	...	...	6·11	6	−0·11	0·002

$$x^2 = 6·549 \qquad N = 8 \qquad P = 0·5 - 0·7$$

This result was confirmed by another worker, who obtained the following results: —$x^2 = 6·571$, $N = 7$, $P = 0·3 - 0·5$.

TABLE XI

THE ERRORS OF COUNTING TUBES ON SUCCESSIVE OCCASIONS BY METHOD (III)

Counts			Variance	Counts			Variance
183	190	187	12·5	238	236	240	4·0
208	206	211	6·5	218	214	219	7·0
201	205	193	37·5	216	211	215	7·0
209	207	207	1·5	237	234	244	26·5
210	206	208	4·0	224	224	218	12·0
182	175	180	13·0	230	233	228	7·0
209	209	210	0·5	200	202	201	1·0
206	205	202	4·5	188	189	188	0·5
203	203	203	0·0	205	200	207	13·0
221	218	218	3·0				
232	231	229	2·5				

Overall Variance = 8·175
Standard Deviation = 2·86
S.E. of Mean of Three Counts = 1·65

TABLE XII

THE EFFECT OF TEMPERATURE AND COMPOSITION OF MEDIUM ON THE VIABLE COUNT

	Medium A					Medium B					Medium C				
	Incubation Temperature					Incubation Temperature					Incubation Temperature				
	20°C	26°C	32°C	37°C	42°C	20°C	26°C	32°C	37°C	42°C	20°C	26°C	32°C	37°C	42°C
	595	575	659	609	591	544	553	620	601	614	416	531	403	499	306
	644	610	679	608	603	596	566	706	616	596	325	507	403	504	319
	621	550	660	567	603	555	574	616	657	650	308	498	452	429	391
	636	583	658	657	631	591	586	572	564	653	321	413	414	501	398
	658	541	693	574	591	644	564	659	663	—	365	549	433	481	396
Mean ...	631	572	670	603	614	586	569	635	620	628	347	499	421	483	362

At 18 hours many colonies are too small easily to be seen. At 48 hours large colonies show " tailing." At 24 hours colonies are all discrete and can be distinguished with ease.

TABLE XIII

GOODNESS OF FIT OF χ^2 OBTAINED FROM 20 REPLICATE ROLL-TUBES OF
BACT. LACTIS AEROGENES

Count (x)	Mean ($\bar{x}$)	$(x - \bar{x})$	$(x - x)^2$	$\chi^2 = \dfrac{S(x - x)^2}{\bar{x}}$
220		9	81	
230		19	361	
224		13	169	
197		14	196	
203		8	64	
208		3	9	
191		20	400	
177		34	1156	
244		33	1089	
209		2	4	
201		10	200	$\dfrac{4705}{211} = 22 \cdot 3$
228	211	17	289	
205		6	36	
235		24	576	
207		4	16	
207		4	16	
209		2	4	
202		9	81	
218		7	49	
208		3	9	
	$\chi^2 = 22 \cdot 3$ N = 19 P = $0 \cdot 2 - 0 \cdot 3$			

TABLE XIV

EFFECT OF DURATION OF INCUBATION ON THE VIABLE COUNT OF *BACT. LACTIS AEROGENES*

Period of Incubation	Count					Mean	Variance
18 hours	34	43	37	40	36	38	50
24 hours	32	41	37	40	36	37	51
48 hours	32	42	38	38	36	37	53

TABLE XV

GOODNESS OF FIT OF VALUES OF χ^2 OBTAINED FROM COUNTS ON SETS OF
FIVE ROLL-TUBES USING *BACT. LACTIS AEROGENES*

Value of χ^2	Expected frequency (m)	Observed frequency (m + χ)	Difference (χ)	$\dfrac{\chi^2}{m}$
Under 1	8·48	11	2·52	0·75
Between 1 and 2	16·36	20	3·64	0·81
,, 2 and 3	16·72	17	0·28	0·01
,, 3 and 4	14·26	13	−1·26	0·11
,, 4 and 5	11·15	10	−1·15	0·12
,, 5 and 6	8·26	4	−4·26	2·20
,, 6 and 7	5·94	5	−0·94	0·15
,, 7 and 9	7·03	9	1·97	0·55
Over 9	5·74	5	−0·74	0·10

$$x^2 = 4·80. \qquad N = 8. \qquad P = 0·7\text{—}0·8.$$

TABLE XVI

THE NON-DESTRUCTIVE ACTION OF QUARTER-STRENGTH RINGER'S SOLUTION ON
BACT. LACTIS AEROGENES

	Period of Exposure (Minutes)				
	0	30	60	120	180
Replicate Counts	416	398	405	338	398
	403	331	365	402	418
	422	359	383	363	357
	407	406	402	431	364
	400	390	388	367	408
Mean...	416	377	389	392	389

RESULTS WITH DRIED POWDERS

The Preparation of the Spray-Dried Powders. The technique of spray-drying and its use for the preparation of powders containing viable organisms have been described by Bullock and Lightbown[5]. In the present work the substrate used was 4 per cent. peptone water. This was adjusted to pH 7·6, filtered distributed in bottles of 1-1. capacity and sterilised by autoclaving. The contents of the bottles were inoculated with a quantity of the suspension of the test organism (spore suspension in the case of *B. subtilis*) calculated to give rise to a count of approximately 2×10^5 per ml. of peptone water. The resultant suspension, cooled in ice, was then spray-dried using an air inlet temperature of 180° to 190°C. for *B. subtilis* and of 70° to 80°C. for *Bact. lactis aerogenes*. Free-flowing powders were obtained in each case. These were stored over phosphorus pentoxide in a desiccator.

Reconstitution of the Bacterial Suspension. A weighed quantity of the powder obtained was dissolved in about 9 ml. of diluent (glass-distilled water in the case of *B. subtilis* and quarter strength Ringer's solution for *Bact. lactis aerogenes*). Bullock and Lightbown[5] compared the strength of this reconstituted solution with that of the original by estimating the chloride content of each. In the present case, however, the substrate was

required to be of minimal chloride content for use in subsequent experiments. The two solutions were, therefore, compared by a colorimetric method. The powder was dissolved in such a quantity of diluent as would produce a solution stronger than the original solution from which the powder was prepared. 4 ml. of this reconstituted suspension was transferred by means of a sterile pipette to one cup of a Spekker photoelectric absorptiometer; 6 ml. of the original suspension was placed in the other cup of the absorptiometer. The two solutions were compared using a dark-blue filter and water was measured into the reconstituted suspension until it matched the original. Mixing of the diluted suspension was effected by means of a platinum wire and the extent of the dilution was noted. The remainder of the reconstituted suspension was diluted proportionately with the sterile diluent. During the process of spray-drying some darkening of the peptone may occur. This would affect the colorimetric reconstitution, increasing the dilution of the reconstituted suspension and causing it to have a lower count. This effect was investigated by spray-drying a 4 per cent. solution of peptone containing 0·5 per cent. of sodium chloride. The relative strengths of the original solution and a reconstituted sample were then determined both by the colorimetric method and by estimation of the chloride content. The results are given in Table XVII.

It was considered established that the colorimetric method was sufficiently accurate.

Percentage mortality of the organism on drying. To determine the effects of spray-drying on the organism in suspension viable counts were carried out on the suspensions fed to the dryer and the material reconstituted as described in the previous paragraph. As will be seen from Tables XVIII and XIX, *B. subtilis* spores suffer up to 10 or 12 per cent. mortality, while only 0·05 to 1·6 per cent. of the *Bact. lactis aerogenes* survive.

TABLE XVII

	Weight Taken (g.)	Volume of Water (ml.)	Colorimetric Factor	Back Titre of Ammonium Thiocyanate Solution	Titre of Sample	Chemical Factor	Error of Colorimetric Method
Original ...	—	—	1·000	5·5 ml.	15·55 ml.	1·000	—
Sample 1 ...	0·7777	15·0 ml.	1·000	3·2 ml.	14·65 ml.	0·945	−5·5 per cent.
Sample 2 ...	0·7563	14·0 ml.	1·175	1·8 ml.	17·45 ml.	1·123	−5·4 per cent.

20 ml. of Silver Nitrate Solution ≡ 21·05 ml. of Ammonium Thiocyanate Solution

TABLE XVIII

PERCENTAGE OF *B. SUBTILIS* SPORES (SUSPENDED IN 4 PER CENT. PEPTONE WATER) KILLED BY SPRAY-DRYING AT VARIOUS TEMPERATURES

Temperature of Inlet Air	Percentage Mortality	Temperature of Inlet Air	Percentage Mortality
134°C.	12·2	165°C.	0·0
150°C.	0·76	181°C.	3·6
150°C.	11·7	205°C.	0·0

TABLE XIX

MORTALITY OF *B. LACTIS AEROGENES* (SUSPENDED IN 4 PER CENT. PEPTONE WATER)
DURING SPRAY-DRYING AT VARIOUS TEMPERATURES

Temperature of Inlet Air	Percentage Mortality	Temperature of Inlet Air	Percentage Mortality
70°C	98·40	120°C.	99·95
75°C	99·38	150°C.	99·68
75°C	99·37	180°C.	99·61
80°C	99·90		

The Distribution of Organisms in the Powder.—To test the distribution of the organisms in the powders obtained by spray-drying, 10 samples of powder were weighed out and dissolved in quantities of diluent proportional to their weights, so that the resultant solutions contained equal concentrations of peptone. The weights of powder taken were such that the solutions obtained from them gave a count of about 200,000 per ml. The 10^{-3} dilution from each sample was then plated out in quintuplicate and the counts determined, as described in the earlier part of this paper. These were compared by means of the Analysis of Variance. The counts obtained with *B. subtilis* are recorded in Table XX and the Analysis of Variance of these in Table XXI.

The results for a similar experiment using *Bact. lactis aerogenes* are given in Table XXII and the corresponding Analysis of Variance in Table XXIII.

TABLE XX

QUINTUPLICATE PLATING OF 10 SAMPLES OF THE SAME SPRAY-DRIED POWDER
(*B. SUBTILIS*)

Sample	I	II	III	IV	V	VI	VII	VIII	IX	X
Weight (g.)	·5198	·5615	·5311	·4954	·5131	·6555	·5295	·4860	·6582	·5095
Volume (ml.)	8·8	9·5	9·0	8·4	8·7	11·1	8·95	8·2	11·2	8·65
	249	224	232	210	228	233	252	230	247	247
	230	238	236	247	241	256	264	253	226	231
Counts	260	227	211	244	233	205	227	232	230	241
	234	227	223	245	220	246	248	255	223	224
	238	262	233	207	278	216	239	233	236	214
Total Counts	1211	1178	1135	1153	1200	1156	1230	1203	1162	1157

TABLE XXI

ANALYSIS OF VARIANCE OF QUINTUPLICATE PLATING OF 10 SAMPLES OF THE
SAME SPRAY-DRIED POWDER (*B. SUBTILIS*)

Source of Variation	Sum of Squares	N	Mean Square	Variance Ratio	P
Difference between samples ...	1710·9	9	190·1	1·296	>0·2
Difference between individuals (error) ...	9859·6	40	246·5		
Total ...	11570·5	49			

Table XXIII suggests that the organisms are not very evenly distributed in the powder. A further spray-drying was performed and samples taken and counted as before. The results are given in Table XXIV and the Analysis of Variance in Table XXV.

TABLE XXII

QUINTUPLICATE PLATING OF 8 SAMPLES OF THE SAME SPRAY-DRIED POWDER
(*BACT. LACTIS AEROGENES*)

Sample	I	II	III	IV	V	VI	VII	VIII
Weight (g.) ...	·5284	·4680	·5044	·4991	·5061	·4331	·4037	·5702
Volume (ml.) ...	13·21	11·7	12·61	12·48	12·4	10·83	10·09	13·01
Counts	160 181 163 180 180	191 163 173 158 163	151 149 152 140 154	183 187 177 152 188	142 154 146 148 158	171 166 141 150 163	198 138 167 189 184	178 149 149 134 159
Total Counts	864	848	746	887	748	791	876	759

TABLE XXIII

ANALYSIS OF VARIANCE OF QUINTUPLICATE PLATING OF 8 SAMPLES OF THE
SAME SPRAY-DRIED POWDER (*BACT. LACTIS AEROGENES*)

Source of Variation	Sum of Squares	N	Mean Square	Variance Ratio	P
Difference between samples	4100	7	585·7	2·505	0·01—0·05
Difference between individuals	7482	32	233·8		
Total	11582	49			

Table XXV shows that the organisms in the powder obtained from the drying of a suspension of *Bact. lactis aerogenes* were distributed very unevenly. The remainder of the powder was therefore placed in a sterile vaccine bottle together with some sterile glass beads. The bottle was closed by means of a rubber cap and fixed to a revolving wheel by means of which it was slowly rotated for a period of 24 hours. 10 further samples were then taken and viable counts performed as before. These are given in Table XXVI and the Analysis of Variance in Table XXVII.

TABLE XXIV

QUINTUPLICATE PLATING OF 10 SAMPLES OF THE SAME SPRAY-DRIED POWDER
(*BACT. LACTIS AEROGENES*)

Sample	I	II	III	IV	V	VI	VII	VIII	IX	X
Weight (g.) ...	·3170	·2600	·2345	·2555	·2075	·2160	·2355	·1830	·2215	·3305
Volume (ml.) ...	9·0	7·4	6·65	7·25	5·9	6·15	6·7	5·2	6·3	9·4
Counts	53 59 36 52 53	54 86 71 77 71	55 47 54 53 60	40 38 48 49 31	46 51 43 56 53	58 53 49 44 74	53 45 55 49 61	35 39 34 45 51	42 41 49 44 64	35 45 46 46 44
Total Counts ...	253	359	269	206	249	278	263	204	240	207

TABLE XXV

ANALYSIS OF VARIANCE OF QUINTUPLICATE PLATING OF 1C SAMPLES OF THE
SAME SPRAY-DRIED POWDER (*BACT. LACTIS AEROGENES*)

Source of Variation	Sum of Squares	N	Mean Square	Variance Ratio	P
Difference between samples	3825·5	9	425·05	4·71	<0·01
Difference between samples	3604·8	40	90·12		
Total	7430·3	49			

TABLE XXVI

QUINTUPLICATE PLATING OF 10 SAMPLES OF THE SAME SPRAY-DRIED AND
MIXED POWDER (*BACT. LACTIS AEROGENES*)

Sample	I	II	III	IV	V	VI	VII	VIII	IX	X
Weight (g.) ...	·2385	·2640	·2570	·2605	·3000	·2895	·2870	·3850	·4230	·3250
Volume (ml.) ...	7·0	7·75	7·55	7·65	8·8	8·5	8·4	11·3	12·4	9·55
Counts	76 80 95 80 85	91 86 89 71 94	91 106 93 110 112	109 89 119 76 94	56 92 95 88 98	82 93 100 91 72	8_ 103 106 112 97	112 114 72 97 86	91 102 104 89 105	88 88 95 118 71
Total Counts ...	416	431	512	487	429	438	499	481	491	460

TABLE XXVII

ANALYSIS OF VARIANCE OF QUINTUPLICATE PLATING OF 10 SAMPLES OF THE
SAME SPRAY-DRIED POWDER (*BACT. LACTIS AEROGENES*)

Source of Variation	Sum of Squares	N	Mean Square	Variance Ratio	P
Difference between samples	2097·9	9	233·10	1·184	>0·2
Difference between individuals (error)...	7877·4	40	196·93		
Total	9975·3	49			

Tables XXVI and XXVII show that after thorough mixing by the above method the organisms have become evenly distributed in the powder.

The Viability of Organisms in Stored Powders.—The powders obtained by spray-drying were stored over phosphorus pentoxide in desiccators at room temperature. Samples were weighed out at intervals and dissolved in quantities of diluent proportional to their weight. The resultant suspensions were suitably diluted and the final dilutions were plated out in quintuplicate (10^{-3} for *B. subtilis*, 10^{-2} for *Bact. lactis aerogenes*). The mean count was determined for each set of 5 tubes and these means are set out in Table XXVIII.

TABLE XXVIII

 EFFECT OF STORAGE ON COUNT OF ORGANISMS CONTAINED IN SPRAY-DRIED POWDERS

B. subtilis							
Period of Storage (Days)	0	12	22	48	80	124	157
Mean Count of 5 Tubes	129	124	126	132	131	132	128

Bact. lactis aerogenes							
Period of Storage (Days)	0	6	7	9	13	21	37
Mean Count of 5 Tubes	5426	743	513	309	301	200	64

DISCUSSION

Viable counts have always been subject to much criticism. In the last few decades some of the objections have been overcome. As a result of carrying out a large number of counts and submitting the results to statistical analysis it has been shown that, with certain organisms and using particular techniques, counts may be performed so as to give results reproducible within certain ascertained limits of error. Methods have also been elaborated for establishing the suitability or otherwise of particular media. In the present work *B. subtilis* spores and *Bact. lactis aerogenes* non-sporing organisms have been submitted to this type of examination.

Tables V to X show that the medium used (Medium A, Table V), is suitable for *B. subtilis* and that the spores can be counted accurately, the errors involved being no greater than the normal errors of random sampling. Having shown that satisfactory viable counts of *B. subtilis* spores could be performed if the spores are in the form of an even suspension, the next task was to examine the spray-dried powder containing these spores. Table XVIII shows two things. In the first place the mortality on drying is low and in the second place variations in the conditions of drying, e.g., in the temperature of the inlet air or the rate of flow of the liquid, have comparatively little effect on the percentage of organisms surviving. Thus one might expect to obtain an even distribution of spores in the resultant powder since local conditions at the jet, or variations in the length of time the powder is lodged in the machine, would not be expected greatly to alter the count of different portions of the powder. Further, in a previous paper it was shown that spores in dry powders were resistant to comparatively high temperatures. It has also been shown that the spores remain viable in the resultant powder over considerable periods of time and that there is little, if any, diminution in the viable count of the powder (Table XXVIII). That the expected even distribution of spores in the powder is in fact obtained is proved in Table XXI. The count variation from sample to sample of powder is shown statistically to be accounted for by the normal errors of random sampling. It is clear from the conclusions established that spray-dried powders containing *B. subtilis* spores are suitable for use

in further experiments. Such experiments involving the exposure to anti-
septics and heat of both the powder itself and the powder suspended
in oil, are in progress in this department.

In the case of *Bact. lactis aerogenes* the circumstances are not so simple.
Table XIX shows that between 98·4 and 99·9 per cent. of the organisms
are killed, i.e., that 0·1 to 1·6 per cent. survive, a very great variation.
Furthermore, Table XXVIII shows that the organisms die off rapidly in
the dry powder, a result previously reported by Bullock and Lightbown[5],
who also showed that *Bact. lactis aerogenes* even in powder form is con-
siderably more sensitive to heat than the spores of *B. subtilis*. Thus we
should expect that slight variations in drying conditions around the spray
jet and in the time during which the various portions of powder are in
contact with the metallic surfaces of the drying chambers would have a
considerable effect on the number of organisms surviving. It is not there-
fore surprising to find that as shown in Tables XXIII and XXV there is
evidence of uneven distribution of the organisms in the dried powder as
discharged from the dryer. However, the powder is light and easily
mixed and this treatment is shown in Table XXVII to result in a powder
in which the organisms are evenly distributed.

It would appear, therefore, that the satisfactory nature or otherwise of
a spray-dried powder probably depends upon the resistance of the
organism to the drying process. If the organism is resistant, as in the case
of *B. subtilis* spores, the powder is eminently satisfactory for further
work. If the organism is susceptible as in the case of *Bact. lactis aero-
genes*, then the powder is not so suitable. It requires careful mixing and
in examining the effects of heat or antiseptics on the powder, and powder
suspended in oil, the high death rate normally associated with the
organisms must be taken into account. Experiments are therefore in
progress to see if a more suitable organism than *Bact. lactis aerogenes*
can be found and some, more promising, results have been obtained with
Streptococcus faecalis. Meanwhile it can be said that a powder containing
Bact. lactis aerogenes in even distribution can be obtained and is suitable
for use in experiments concerned with the study of environmental condi-
tions on a relatively sensitive organism.

SUMMARY

1. The technique of performing viable counts using graduated pipettes
with roll-tubes has been examined and the accuracy of the method has
been assessed by statistical analysis.

2. It has been shown that satisfactory viable counts can be obtained
of spores of *B. subtilis* in suspension or in powders. The spreading surface
growth of the organism has been shown to have no significant effect upon
the count and roll-tube counts may be performed satisfactorily in its
presence.

3. It has been shown that if even suspensions of *B. subtilis* spores are
spray-dried the viable organisms are evenly distributed in the resultant
powder.

4. It has been shown that if even suspensions of *Bact. lactis aerogenes* are spray-dried the viable organisms are not evenly distributed in the powder as taken from the spray-drier, but that an even distribution can be obtained if the powder is thoroughly mixed by mechanical means.

5. The effect of storage on the viability of the organisms in peptone powders has been examined. The viable count of *B. subtilis* was found to undergo no significant diminution after 6 months' storage, whereas the viable count of *Bact. lactis aerogenes* fell rapidly, most of the organisms dying within the first few days.

6. It is concluded that spray-dried powders containing spores of *B. subtilis* are very suitable for examining the effects of environmental conditions on the spores. On the other hand, the use of powders containing organisms, such as *Bact. lactis aerogenes,* which show a high mortality on spray-drying, involves greater difficulties, which may be inherent in the problem.

It is a pleasure to express our thanks to Professor M. S. Bartlett for suggestions and advice concerning the statistical treatment of the results reported in this paper.

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DISCUSSION

Miss Winifred Keepe presented the paper.

MR. B. A. BULL (Nottingham), deputising for the Chairman, said that the work described was an extension of the valuable work of Dr. Bullock and his colleagues on spray drying. The authors seemed to have evolved a suitable technique for a complex subject.

DR. K. R. CAPPER (London) said that bacteria in dust were in an environment of low moisture content, and dust was a very probable

source of infection. Apparently a greater number of cells developed from a medium of lower food content. It might be advisable to use a peptone medium containing yeast extract and not meat extract. With *B. subtilis* he had found greater or more consistent growth in liquid media containing yeast extract, and, in particular, in yeast autolysate dextrose media. The addition of manganese sulphate tended to give a more consistent growth. The addition of such substances might not increase the number of cells which actually grew to produce colonies; but he would like to know whether the authors had tried yeast extract or any type of yeast media or the addition of trace elements. When cells were under favourable conditions certain parts of their enzyme systems were likely to be destroyed or damaged before others, and the composition of the medium used was then of considerable import-ance. He did not think that it was certain that the type of medium used in sterility tests was the best, or that the type of control using diluted 24-hour cultures was necessarily the best type of control. Cells which had been damaged by drying, for instance, might not grow, whereas fully viable cells would grow. The time of incubation was 60 hours, but spores were liable to become dormant and to develop in much longer periods; he believed that Dr. Davis had found that certain spores developed after some weeks. There was considerable difficulty in keeping solid cultures in aga media for that length of time, though there were techniques for doing this.

Dr. I. MICHAELS (London) said that the authors by treating their results statistically had brought their work into line with that of other contributors in this field. Two important factors had been studied; the first was the behaviour of the sporing organism on a roll tube, and the second was the use of volume tests for accurate dilutions. The standard error of the mean of the three counts in Table III, Method (3), namely, 2·94, was well within the accepted limit of $\pm$ 5 per cent. The number of organisms on the tubes was rather high, and must have been responsible for much tedious work. The rating of volumes was far less troublesome than counting numbers of groups, but the choice of tech-niques depended largely on the number of organisms. For compara-tively small numbers, such as the hundreds of thousands which the authors had employed, the degree of dilution was not high. However, when hundreds of millions of organisms were used, as when dealing with bactericidal activity of disinfectants, the degree of dilution was con-siderable, and a technique which did not allow a high dilution at each stage, coupled with the employment of small but accurately determinable quantities, would render the overall technique unmanageable.

Mr. G. R. MILNE (Glasgow) asked what was the moisture content of the powders prepared by spray drying and stored, he thought, over phosphorus pentoxide, and what was the likely effect of small variations in this moisture content on the organisms in the powder. For dried

human plasma, prepared by a freeze drying process, what would be the effect of bad sealing, and perhaps bad storage, on the content of bacteria.

MR. R. MAXWELL SAVAGE (Barnet) asked whether any of the experiments described had been extended to the anærobic sporing organisms, because there was a very interesting ecological point involved. In an attempt which he made some years ago to prepare an artificial powder containing anærobic spores he found that they died off with extreme rapidity.

DR. K. BULLOCK, in reply, said that, although the authors had adopted statistical techniques in the present paper, they had not yet become firm and consistent worshippers of statistics; in fact, so far they had not used them, in the sense of coming to any conclusion as a result of their statistics which would not have been formed from a simple inspection of the figures. The statistical analysis, however, would be necessary later on, when they had to decide such matters as whether a count had not fallen at all or had fallen slightly. In any case the present paper was a preliminary one. He thanked Dr. Capper for his remarks on sensitive media. They know that some of the cells were bound to be damaged, but they had incubated for a long time and had tried as far as possible to take the matter into consideration. It was, however, something which would have to be carefully reconsidered at various stages as the research developed. The moisture content of the powders in question when dried over phosphorus pentoxide was fully described in a previous paper by Mr. Wright and himself, where they stored these powders, having exposed them to various aqueous pressures, and then recorded the fall or otherwise of the counts. They had not dealt with the anærobes, because the present type of spray dryer was not suitable; it would be necessary to spray in a current of nitrogen.

MISS KEEPE, replying to Dr. Michaels with regard to the number of organisms, said that they chose between 200 and 400 because that number had been reported by previous workers to be the most suitable number.

PROTEIN IN MALTED PREPARATIONS

BY F. WOKES AND CHLOE KLATZKIN

From the Ovaltine Research Laboratories, King's Langley, Herts

PREVIOUS communications[1,2,3,4] from these laboratories have dealt with the general food value of malted preparations, particularly malted barley and malt extract, giving data on the content of B vitamins (aneurine, nicotinic acid and riboflavine), of carbohydrates and of " protein." The present communication deals with the nature of the nitrogenous constituents included in the term " protein."

The nitrogenous constituents of malt extract have received poor recognition from official sources. The British Pharmacopœia, 1932, specified a minimum protein content of 4·5 per cent. based on 6·25 times total N_2, but the British Pharmaceutical Codex, 1934, attributed the nutritive action of malt extract to carbohydrates and B vitamins, ignoring the protein, and the United States Pharmacopœia XII also ignored protein as a constituent. Since the malt extract of U.S.P. XII also contained 10 per cent. of glycerin, giving a more readily fermentable product for which sterilised containers had to be prescribed, it was perhaps fortunate that it was excluded from the U.S.P. XIII. The 4·5 per cent protein minimum of the B.P. 1932, can be considerably exceeded in a good malt extract, as one of us (F.W.) showed in 1943. Nevertheless, the protein minimum in the B.P. 1948 has been lowered to 4·0 per cent.. A moderate diastatic value was introduced and then almost immediately withdrawn. Previous workers[5] have recommended the use of malt extracts with very low diastatic values for malt and oil manufacture, but this would involve the loss of protein and B vitamins. We hope to show that the proteins of malt extract and of other malted preparations are of greater importance than these pharmaceutical divagations indicate.

METHODS

Total nitrogen (micro-method). A quantity of material containing about 3 mg. of N_2 was heated with 2 ml. of concentrated sulphuric acid and a trace of mercuric sulphate and 50 mg. potassium sulphate for half an hour after charring had been completed. The cooled solution was placed in a micro-Kjeldahl flask, 10 ml. of 40 per cent. sodium hydroxide solution and 1 ml. of 40 per cent. sodium sulphide solution were added and the contents of the flask were distilled with steam into 10 ml. of N/20 sulphuric acid. The latter was boiled to remove carbon dioxide and titrated against N/20 sodium hydoxide using methyl red as indicator. The difference betwen the reading and that given by a blank with reagents only represented the nitrogen from the sample. The micro method was employed to estimate minute amounts of nitrogenous constituents in germinated seeds of which only small quantities were available. It was controlled against ammonium sulphate and gave good agreement with the B.P. 1948 method.

Salt soluble nitrogen. The method of the Association of Official

Agricultural Chemists[6] was used with 1 per cent. of sodium chloride instead of 5 per cent. sodium sulphate, to minimise bumping during concentration of solutions. This alteration made no difference to the results.

Non-protein nitrogen. The method of the Association of Official Agricultural Chemists[6] was used.

Amino acids were estimated chemically by the methods described by Block and Bolling[7]. Some of these (e.g. for tryptophane) did not give very reliable results and our findings with them must be considered only tentative. However, with arginine, one of the most important aminoacids in our materials, we used Dubnoff's[8] modification of Sakaguchi's method[9], and obtained on a series of oats samples good agreement with results obtained at the Cereals Research Station, St. Albans, by Mr. J. G. Heathcote, who has had considerable experience of this problem, and to whom we are indebted for advice and help.

Aneurine was estimated fluorimetrically by the method[10] previously described which has given good agreement with microbiological methods.

Nicotinic acid was estimated colorimetrically using either *para*-aminoacetophenone[11] or *para*-aminopropiophenone[12] as the aromatic amine. Both of these gave satisfactory agreement with microbiological assays.

Riboflavine was estimated fluorimetrically by a method[13] giving good agreement with microbiological assays.

Diastatic index was estimated as previously described[1].

Trypsin inhibitor of soya and other foods was estimated by a modification of the method of Bowman[14] using skimmed milk instead of casein as substrate, and following the process of digestion by formol titrations.

RESULTS

Differentiation of nitrogenous constituents. (a) *Total nitrogenous constituents.* The B.P. 1948 method estimates total nitrogen by the Kjeldahl method and multiplies the result by 6·25 to determine the protein content of malt extract. This involves two faulty assumptions—that the factor 6·25 is accurate for cereal proteins, and that the nitrogenous constituents are all protein. We have overcome both these difficulties by quoting our results as nitrogen instead of as protein content. On this basis Extract of Malt B.P. 1948 should contain at least 0·64 per cent. of total nitrogen. This seems quite a low limit. Our results on 37 samples from 13 manufacturers, summarised in Figure 1, show that only two samples fell significantly below this lower limit, but seven samples fell below the B.P. 1932 limit. Our own samples were all well above this higher limit. Confirmation was provided of our previous finding that a low total nitrogen is usually accompanied by a low diastatic value. Data on malted barleys also given in Figure 1 show a similar tendency for the total nitrogen content to fall with decrease in diastatic value. However, we think that the wide variation in the total nitrogen of malt extracts is probably due partly to differences in brewing conditions.

(b) *Protein nitrogen* (from albumin and globulin plus " less soluble " protein). Our results in Table I show that only 12 to 44 per cent. of the total nitrogen content of malt extract represents true protein, as distinct from protein digestion products.

(c) *Salt-soluble nitrogen.* This figure includes nitrogen from albumins and globulins as well as from the breakdown products of these and other proteins, all of which are important in infant feeding. It therefore provides valuable information on the nutritive value of malted preparations. Our results (see Table I) show that it can vary significantly in different samples.
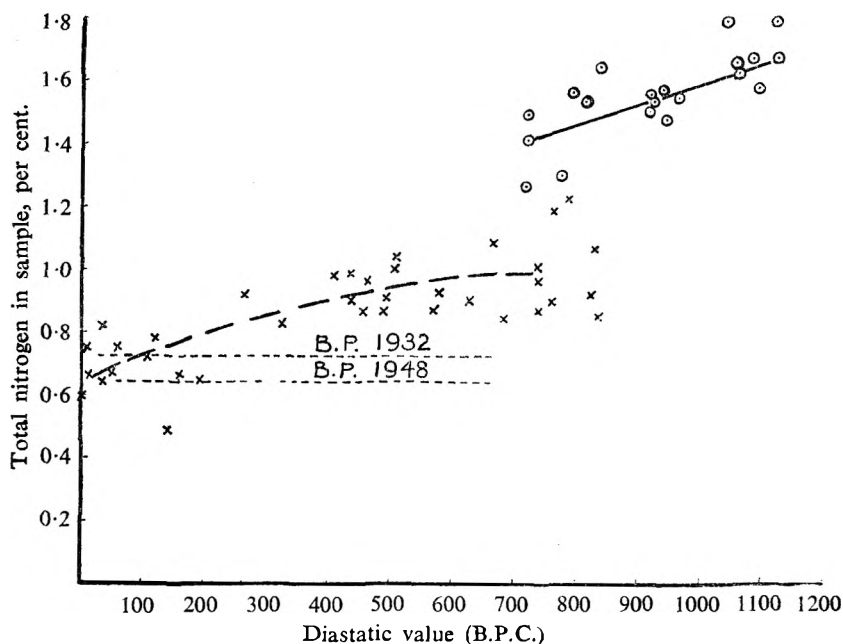

FIG. 1. Relation between diastatic value and total nitrogen content of malted barley (indicated thus ⊙——⊙) and of malt extract (indicated thus X— —X).

(d) *Non-protein nitrogen.* This figure includes the soluble breakdown products of the cereal proteins, with possible slight traces of asparagine and glutamine (which are important in plant metabolism[15] but have yet

TABLE I

DIFFERENTIATION OF NITROGENOUS CONSTITUENTS IN MALT EXTRACT

Sample				Salt-soluble nitrogen	" Non protein " nitrogen	Albumin and globulin nitrogen	" Less soluble " nitrogen	Total nitrogen
3 C	...	...	...	...	1·60	0·98	0·62	0·08	1·68
3 B	...	...	...	...	1·47	0·98	0·49	0·13	1·60
3 D	...	...	...	...	0·94	0·57	0·37	0·07	1·01
9 B	...	...	...	...	1·10	0·87	0·23	0·03	1·13
9 A	...	...	...	...	0·93	0·72	0·21	0·06	0·99
9 C	...	...	...	...	0·93	0·72	0·21	0·05	0·98
5 A	...	...	...	...	0·815	0·73	0·085	0·015	0·83
7 A	...	...	...	...	0·74	0·56	0·18	0·08	0·82
8 A	...	...	...	...	0·72	0·53	0·19	0·08	0·80
3 A	...	...	...	...	0·51	0·40	0·11	0·03	0·54

Notes —Albumin and globulin nitrogen = salt-soluble − non-protein nitrogen.
" Less soluble " nitrogen = total nitrogen − salt-soluble nitrogen.

to be established as significant in animal metabolism). The figure provides a useful measure of the extent of enzyme action during malting and brewing.

(e) *"Less soluble" nitrogen.* This figure represents the structural protein of plants, but would also include protein coagulated by heat during manufacturing processes. Little is yet known about its degree of availability to infants or its importance in infant nutrition. Our results in Table II show that it can vary widely in different samples, and we would suggest that preference be given to malted preparations in which the " less soluble " nitrogen has been reduced to a low level.

TABLE II

PERCENTAGE DISTRIBUTION OF NITROGENOUS CONSTITUENTS IN TOTAL NITROGEN IN MALT EXTRACT

Sample						Percentage of total nitrogen represented by nitrogen from		
						Albumin and globulin	Predigested protein	" Less soluble " protein
3 C	...	...	...	...	...	37	58	4·8
3 B	...	...	...	...	...	31	61	8·1
3 D	...	...	...	...	...	37	56	6·9
9 B	...	...	...	...	...	20	77	2·7
9 A	...	...	...	...	...	21	73	6·0
9 C	...	...	...	...	...	21	74	5·1
5 A	...	...	...	...	...	10	88	1·8
7 A	...	...	...	...	...	22	68	9·8
8 A	...	...	...	...	...	24	66	10·0
3 A	...	...	...	...	...	20	75	5·5

Protein value of malted preparations in infant feeding.

The experience of one of us (C.K.) shows that malted preparations are widely employed in London hospitals as dietary supplements for young children, especially those who are undernourished. Whilst some of the nutritive virtues of these malted preparations are doubtless due to their vitamin content, the presence of readily assimilated protein and carbohydrate must be of value. As long ago as 1865 Liebig[16] showed by experiments on his breast-fed grandsons that a deficiency in the supply of human milk could be made good by " Malzsuppe," a malted infant food prepared by heating an aqueous extract of malted barley with wheat flour and milk. The milk provided about one-third of the total solids and two-thirds of the total protein. This led to the introduction of malt extract for infant feeding, and perhaps provided the inspiration for the Italian brewing chemist, Caprino, to devise during wartime emergencies milk substitutes based on malted cereals. In Italy oats are a staple cereal, and hence were used by Caprino, leading to the name " Maltavena." Ward Perkins[17], a British Red Cross worker interested in the Italian experiments passed on the idea to U.N.R.R.A. in London and experiments were begun in England. Here oat flour was replaced by malted barley and wheat flour, and soya flour was added to improve the protein value, but the name " Maltavena " was still retained. A series of experimental batches were made in our laboratories, and tested clinically on babies and physiologically on rats. The clinical tests were

not sufficiently comprehensive to show more than that babies could tolerate Maltavena. The physiological tests, carried out at Cambridge by Chick and Slack[18], showed that some of the samples closely resembled milk in their growth-promoting properties.

In view of these physiological results it was decided to institute large scale clinical trials in Germany. We prepared some of the material used in these trials, sending over a ton to Germany during 1947. The results of these large-scale clinical trials have not yet been published. However, a few clinical tests of our materials have also been made in this country. Sufficient data is not yet available to permit publication of precise figures, but the indications are that malt and soya baby foods containing about 10 per cent. of " protein " (based on a total nitrogen content of 1·67 per cent.) are inadequate for satisfactory growth. Better results have been obtained with these foods containing about 12 per cent. of " protein " (based on a total nitrogen content of 2·08 per cent.). At this point, however, a new factor came into view, viz. the occurrence in raw soya of a trypsin inhibitor[14] which might affect the digestibility of the Maltavena protein *in vivo* and thus its availability for growth promotion. On applying *in vitro* tests to the soya used as raw material we found that it contained the trypsin inhibitor, which could be removed by acid extraction and destroyed by autoclaving or enzyme action. Moreover, the Maltavena which we had sent to Germany for the clinical trials also contained the inhibitor. Our method of testing for the inhibitor did not permit its precise evaluation using milk as substrate. Such evaluaton was effected by Dr. Borchers of Nebraska University, a leading American investigator in this field, who very kindly examined some of our Maltavena samples and confirmed that the inhibitor was present (see Table III).

TABLE III

TRYPSIN INHIBITOR IN SOYA AND IN MALT AND SOYA FOOD

Materials examined	Inhibitor found by	
	Borchers	Ovaltine Research Laboratories
Soya beans		+++
Soya meal, defatted	100	+++
,, ,, full fat...		+++
,, ,, heated dry 1 hr. at 100°C.		++
,, ,, autoclaved ½ hr. at 115°C.	0	0
,, ,, digested with papain	0	0
Malt and soya food 1945*	5	
,, ,, 1947	2	trace

Notes—
* Tested physiologically for growth promoting properties by Chick and Slack[18].
  Results show relative amounts of inhibitor found using as substrates hæmoglobin (Borchers, private communication) and milk (O.R.L.). Dr. Borchers does not consider that his results indicate the presence of any significant amount of the inhibitor in the two samples.

It was then decided to make further samples in which the inhibitor would be destroyed by autoclaving. Unfortunately this treatment affected the thermoplastic nature of the product, so that vacuum drying became impracticable, and before this difficulty could be overcome the opportunity had passed for taking part in the further German trials.

Vitamin B in Malt and Soya Baby Foods. Before applying growth

results to measure the relative protein values of different samples of malt and soya foods it is perhaps advisable to consider their content of B vitamins. In contrast with vitamins A, C and D, these were not supplied as supplements in all the clinical trials. Hence, if they were deficient in any of the samples this might have affected the growth results and thus confused the issue. Jeans and Marriott[19] in their well-known textbook on Infant Nutrition, state that when infants are fed on milk substitutes based on soya the diet will need supplementing with B vitamins. Table IV shows the content of aneurine, nicotinic acid and riboflavine as

TABLE IV

COMPOSITION OF MALT AND SOYA BABY FOOD

No. of samples examined	2 *
Total solids per cent. w/v	95·0
Composition of total solids :—	
Total nitrogen per cent.	1·68
carbohydrate ,, ...	84·2
fat ,, ...	2·6
ash ,, ...	2·6
Ca mg./100 g.	559 †
Fe ,, ...	6
aneurine µg./g.	4·6
nicotinic acid ,, ...	100 ‡
riboflavine ,, ...	2·3
percentage of total calories from total nitrogen	10
,, of total calories from soluble nitrogen	6·0

Notes—
   * From over a ton of dried product which had been thoroughly mixed to ensure uniformity. Dr. R. G. Booth's results on the samples were in good agreement with ours.
   † Confirmed by microbiological assays by Dr. F. W. Norris. In calculating percentage of total calories from total nitrogen and from soluble nitrogen allowance was made for lower nitrogen factors of vegetable protein.
   ‡ Most of this comes from soluble calcium salts used in the formula.

µg./g. in the " 10 per cent." sample. These data have now to be compared with the actual requirements of babies, and the amounts in human milk, which are given in Table V on the same basis of µg./g. total solids of diet and of human milk respectively. The figures for aneurine and nicotinic acid in human milk are considerably lower than the suggested requirements, and it seems safer to assume that the latter are more reliable. The nicotinic acid figure especially has been queried by later workers[20]. On this requirement basis our samples probably contained

TABLE V

VITAMIN B VALUE OF MALT AND SOYA FOOD AND ITS RAW MATERIALS

Material	content as µg./g. total solids		
	aneurine	nicotinic acid	riboflavine
Malt and soya food " 10 per cent. protein "*	4·6	100	3
Human milk, solids of	2·4‡	16	2–7·5§
Requirements in diet of baby†	5·5	55	8
Malt extract solids*	2·5 to 4·5	100 to 150	2 to 4·5
Per cent. from raw materials—			
Malt extract	75	95	88
Soya	19	2	7
Wheat	6	3	3—4

   * Results obtained in Ovaltine Research Laboratories. † Calculated from data from U.S.A. National Research Council [22], [23]. ‡ Calculated from data by Knott, Kleiger and Schlutz [20]. § Calculated from data of Marriott and Jeans [21].

enough aneurine, and certainly supplied enough nicotinic acid. They may, however, have been deficient in riboflavine. Later samples have been fortified with this vitamin.

Table V shows the proportion of these B vitamins supplied by the different raw materials. It will be seen that by far the greater part of the vitamins comes from the malt extract. Since commercial malt extracts vary widely in their content of B vitamins, stress is laid on the importance of ensuring that this is as high as possible in any malt extract used in the preparation of malt and soya baby foods. This applies particularly to riboflavine, of which soya is rather a poor source. By taking sufficient care in the malting and brewing processes it is possible to obtain malt extracts which are considerably better sources of this vitamin than soya is.

The nicotinic acid content of malt and soya foods made from malted barley should not present much difficulty, judging by the fairly narrow range in commercial malt extracts. If, however, malted barley were replaced by malted oats, as Caprino originally employed, the nicotinic acid content might be more critical. Unmalted oats contain only about one-tenth as much of this vitamin as unmalted barley[24] and in contrast with the latter, the nicotinic acid content can at least double during germination, but will still be only one-fifth of that in malted barley. A similar objection applies to the use of maize in baby foods.

Another point to be considered is the possible loss of B vitamins during removal or destruction of the trypsin inhibitor in soya. Our experiments indicated that the destruction of the inhibitor by autoclaving would involve losing 10 to 20 per cent. of the aneurine in the soya. Table V shows that this would be only 2 to 4 per cent. of the total aneurine in the " 10 per cent." sample. This loss is insignificant. Much greater losses of aneurine can occur by heat treatment during the preparation of soya flour from soya beans. Removal of the inhibitor by extraction with very dilute acid as used by American workers[25] removed much more aneurine and also a good deal of nicotinic acid and riboflavine.

The trypsin inhibitor of soya may also be destroyed by dry heating at a sufficiently high temperature. Recent work[26] shows that such dry heating may, if carried on long enough (say five hours), cause the protein to be less rapidly digested by the enzymes of the gastro-intestinal tract. The amino-acid lysine seems to be particularly affected.

American workers[26] suggest that with optimal processing conditions 50 to 65 per cent. of the protein in soya should be extractable with water. American samples of soya vary widely in their content of water-soluble protein, some containing practically none. In the nitrogenous constituents of the soya we used in manufacturing our samples about 20 per cent. was water-soluble, which is higher than is indicated by the protein figures for some American samples but well below the optimum. Table II showed that in commercial malt extracts at least 90 per cent. of the nitrogenous constituents are soluble. Since this is a much higher proportion than the corresponding figure for soya, increase in the proportion of the latter in Maltavena would be expected to lower the proportion of soluble in total nitrogenous constituents.

Table II showed that in the total nitrogenous constituents of malt extract 56 to 88 per cent. represented protein digestion products. The corresponding figures in barley before malting are 7 to 8 per cent. and after malting 22 to 33 per cent. according to the efficiency of malting. Our " 10 per cent." sample of malt and soya food gave a figure of 36 per cent., our results showing that the nitrogenous constituents of soya almost entirely come from undigested protein. When the soya was subjected to enzyme treatment to destroy the trypsin inhibitor, a certain amount of the soya protein was also predigested, as shown by the increase to 43 per cent. for the 12 per cent. sample.

Amino-acid composition of proteins used in infant feeding. (a) *Essential amino acids from malted barley and other staple cereals.* The nutritive value of the protein in malted preparations depends on its content of essential amino acids. The available data are too scanty and unreliable to permit precise evaluation, but, it may in general terms be said that the proteins from malted barley and other staple cereals contain more of the sulphur containing amino acids than the protein from soya, and compare fairly well with that from milk[27]. This advantage of cereal over soya protein is particularly well marked with cystine. The protein of malted barley contains about twice as much cystine as that of soya, and that of oats about 3 times. Those of rice, wheat and maize are intermediate. Turning to other essential amino acids, soya has an advantage over cereals as a source of arginine and lysine, in which it compares favourably with milk. Whilst the protein of oats contains as much arginine as that of soya or of milk, it contains only half as much lysine. The protein of malted barley contains rather more lysine than that of oats, but much less arginine. The proteins of maize and wheat are also deficient in these two essential amino acids. Thus it seems clear that in the development of a milk substitute based on malted cereals and soya, the malted cereals can make a valuable contribution to the protein value of the product only if they are carefully selected and blended with suitable proportions of soya.

TABLE VI

COMPARISON OF PROTEIN FROM HUMAN AND FROM COWS' MILK FOR THEIR CONTENT OF ESSENTIAL AMINO ACIDS

Amino acid						Percentage of the amino acid in protein from	
						human milk	cows' milk
Arginine						5·0	4·3
Lysine						7·2	7·5
Tyrosine						5·1	5·3
Tryptophane						1·9	1·6
Phenylalanine						5·9	5·7
Cystine + Methionine						5·4	4·4
Threonine						4·6	4·6
Leucine						10·2	11·3
iso-Leucine						7·6	6·2
Valine						9·9	6·6

Note.—Figures published by Block and Mitchell[28] for the amino-acid content of different samples of human milk show wide variations for arginine, tryptophane, cystine and valine, and to a less extent for isoleucine, which may smooth out some of the apparent deficiencies of cows' milk in these essential amino acids. The baby's requirement of arginine may be greater in the earlier stages of lactation, since colostrum contains more than average human milk. The value of malt and soya food as a source of arginine is considered in Table VII.

(b) *Comparison of human and cows' milk proteins as standards.* The high biological value of the proteins in cows' milk justifies its use as the basis of most baby foods. Comparison of the essential amino acid content of cows' milk protein with human milk protein (see Table VI) shows on the whole remarkably good agreement. There are, however, deficiencies in tryptophane, cystine + methionine, *iso*leucine and valine in cows' milk protein, which have led us to prefer human milk protein as a standard for comparison with malted preparations. (Jeans and Marriott[19] suggest that cows' milk protein may be as much as 20 per cent. inferior to human milk protein.)

(c) *Comparison of " malt and soya" protein with human milk protein.* Table VII compares the content of essential amino acids in the protein

TABLE VII

ESSENTIAL AMINO ACIDS IN PROTEIN OF MALT AND SOYA FOOD AS COMPARED WITH THAT OF HUMAN MILK

Each figure shows the concentration of the given amino acid as a percentage of its concentration in the n the protein of human milk.

Animo-acid	10 per cent. sample*	12 per cent. sample*
Arginine	85	92
Lysine	64	68
Tyrosine	54	60
Tryptophane	74	76
Phenylalanine	83	86
Histidine	77	78
Cystine + Methionine	43	41
Threonine	81	82
Leucine	61	62
iso-Leucine	60	60
Valine	45	44

Note.—* Protein contents of 10 and 12 per cent. expressed as percentage of total calories provided by the protein, allowance being made for the lower factor of cereal proteins when calculating calorie value from total nitrogen.

Results by American workers[7,27] indicate that replacement of malted barley by malted oats might be expected to improve considerably the content of arginine, lysine and cystine plus methionine, largely eliminating the deficiency of the samples in these essential amino-acids. Our results on arginine contents of unmalted oats are in agreement with the American results, and we find no appreciable change in arginine content after malting.

In the " 10 per cent." sample about 47 per cent. of the " protein " came from the soya and about 42 per cent. from the malt extract. In the " 12 per cent." sample the corresponding figures were 57 and 34 respectively.

of two of our batches of malt and soya baby foods used in clinical trials with the content of the same amino acids in human milk protein. In both batches the protein is seen to be markedly deficient in a number of these amino acids. The batch of higher protein content was made from a higher proportion of soya and therefore contains more arginine, lysine and other essential amino acids, and slightly less cystine and methionine, but owing to difficulties in estimating these latter amino acids the figures given for them are very approximate and almost certainly, too low.

(d) *Protein value of " malt and soya" compared with that of human milk.* Before attempting to relate the approximate data in Table VII with the clinical results given by the two samples, we thought it desirable to make some allowance for the total protein content. The protein content of human milk decreases rapidly in the earliest stage of lactation. Data from a large number of subjects summarised in Figure 2, show that by the end of the first month the average protein content has fallen to

about 8 per cent., after which it levels out between 7 and 8 per cent. on a calorie basis. Since milk substitutes are hardly ever given until after the first few weeks of lactation it seems fair to compare them with human milk containing about 8 per cent. of protein (on a calorie basis). This

TABLE VIII

COMPARISON OF MALT AND SOYA FOOD WITH HUMAN MILK AS A SOURCE OF ESSENTIAL AMINO ACIDS

Amino-acid						" 10 per cent." sample	" 12 per cent." sample
Arginine	...	...	...	...	...	106	138
Lysine	...	...	...	...	...	80	102
Tyrosine	...	...	...	...	...	68	90
Tryptophane	...	...	...	...	...	93	114
Phenylalanine	...	...	...	...	...	104	129
Histidine	...	...	...	...	...	96	117
Cystine + Methionine	...	...	...	...	54	62
Threonine	...	...	...	...	...	101	123
Leucine	...	...	...	...	...	76	93
iso-Leucine	...	...	...	...	...	75	90
Valine	...	...	...	...	...	56	66
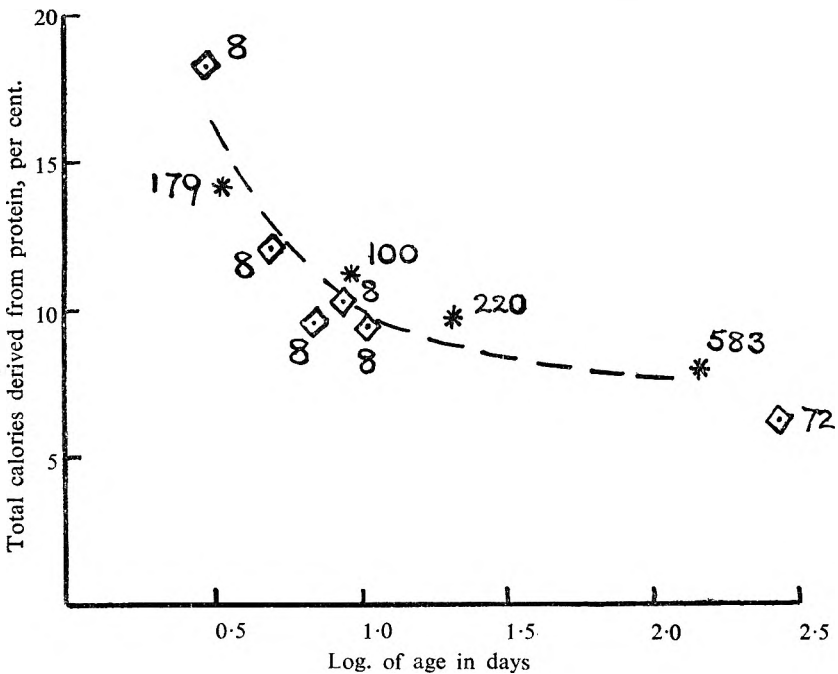
FIG. 2. Relation between protein content of human milk (on calorie basis) and stage of lactation plotted from data published by Gardner and Fox[29] (indicated thus *) and Hammett[30] (indicated thus ◇). Figures against each point refer to number of samples analysed, the majority of these representing all but the first month of lactation gave an average of 8·0.

suggestion is in agreement with Jean and Marriott's[19] findings. An average value of 8 per cent. would imply that in Table VII the figures for

the " 10 per cent." sample could be multiplied by 1·25 and those for the 12 per cent. sample by 1·5 to obtain their amino acid values as compared with human milk. Table VIII shows the effect of doing this. In the 12 per cent. sample all the amino acid values have been brought close to those of human milk, except those for valine, whose importance is perhaps not so great, and for cystine + methionine, for which figures quoted are almost certainly too low. On this basis the protein value of human milk should be definitely superior to that of the 10 per cent. sample, but perhaps not greatly superior to that of the 12 per cent. sample. Moreover, it should be possible to prepare from cows' milk by careful adjustment of the proportion of curd and whey proteins a baby food containing about 8 per cent. of protein (all from milk) which would have a protein value similar to that of human milk. The clinical data so far obtained are in line with these assumptions.

SUMMARY

1. The total nitrogen content of malt extract may range from 0·2 to 1·2 per cent. but is usually well above 0·64 per cent. which corresponds to the B.P. 1948 minimum content of 4 per cent. (total nitrogen x 6·25).

2. Lower diastatic values are usually associated with lower total nitrogen contents, the tendency being observed with malted barley as well as with malt extract.

3. Of the total nitrogen of malt extract only 12 to 44 per cent. is true protein nitrogen. The greater part represents breakdown products of protein, largely formed during malting and brewing.

4. The " less soluble " nitrogen of malt extract may range from 1·8 to 10 per cent. of the total nitrogen, and should be reduced to as low a level as possible in malted preparations.

5. In baby foods prepared from malt extract, wheat flour and soya flour sufficient aneurine and nicotinic acid may be derived from the raw materials if care is taken to use malt extract of high vitamin B content. Fortification with riboflavine may be necessary. If the diet is adequately supplemented by vitamins A, C and D the growth-promoting effect on babies may provide a measure of the protein value of the diet.

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FLUORIMETRIC AND MICROBIOLOGICAL ASSAYS OF RIBOFLAVINE IN MALTED PREPARATIONS

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IN a previous communication[1] we described fluorimetric and microbiological assays of riboflavine in barley, malted barley and malt extract. Whilst the two assay methods gave the same general picture for increases in content of this vitamin during malting and brewing, certain discrepancies were encountered between the results given by the two methods. On 4 samples of barley and malted barley, the fluorimetric method gave lower results (72 to 89 per cent. of the microbiological result), the deviation on 3 of the samples being significant. On 4 samples of malt extract the fluorimetric result did not deviate significantly from the microbiological result. On a fifth sample of malt extract the mean fluorimetric result was 231 per cent. of the mean microbiological result, a highly significant difference. Extensive investigations have been carried out in our laboratories to explain these discrepancies, and show how they may be overcome by improvements in fluorimetric and chromatographic technique.

Elvidge[2] reported good recoveries (84 to 105 per cent.) of this vitamin from 5 more potent pharmaceutical preparations (an elixir, capsules and tablets) in which he estimated it fluorimetrically or spectrophotometrically. A detailed study of the spectrophotometric method was made in our laboratories[3]. Optical densities were recommended to be read at 267 or 375 mμ as well as at the maximum of 444 to 445 mμ recommended by Elvidge. The importance of controlling the pH was emphasised, and experiments described on the development of lumiflavine through the action of light on alkaline solutions of riboflavine. The possible interference of lumiflavine fluorescence has been one of the factors we have had to investigate in the fluorimetric work described below.

MICROBIOLOGICAL ASSAYS

The method as laid down by the Analytical Methods Sub-Committee of the Society of Public Analysts[4] was almost exactly followed. It has been described with comments in a previous paper[1]. Continued experience with the method has shown that *Lactobacillus helveticus* is not on the whole so easy to work with as, for example, *Lactobacillus arabinosus*, which is used for the assay of nicotinic acid and biotin. Difficulties are sometimes experienced in maintaining sub-cultures, and it would seem that the organism requires nutrilites whose nature is not necessarily exactly known, but which are present in some natural products. This is further indicated by the observation in recent months that there is a tendency to obtain less satisfactory linearity in the standard curve than in the curves for the assay samples.

The effect of the presence of fats and fatty acids in samples and

extracts undergoing assay has long been observed, although completely satisfactory explanations are not as yet forthcoming (see, for example, Kodicek and Worden[5], and Norris and Lynes[6]). The occurrence of fats as such in materials for assay need present little difficulty since the sample may be quantitatively pre-extracted with light petroleum in a Soxhlet extractor. The assay is then performed on the fat-free product, and the vitamin content calculated back to the original material. Where the fat or fatty acids occur in lipin combinations, the position is not so simple. Ordinary extraction as above removes little or none of the lipin, and resort to more drastic treatment involves loss of all or part of the vitamin. In these cases it has usually been satisfactory to extract the hydrolysed extracts with light petroleum or, better, ether.

FLUORIMETRIC ASSAYS

We previously employed a modification of the fluorimetric methods of Rubin et al.[7] and of Hoffer et al.[8] kindly given us by Dr. A. J. Amos, Secretary of the Chemical Panel of the Vitamin Sub-Committee of the Society of Public Analysts. Whilst this method as adapted for use with the Spekker fluorimeter gave reasonable agreement with microbiological assays on some foods fairly rich in the vitamin and relatively free from interfering substances, the Panel members did not find it satisfactory for malted preparations and certain other foods in which more of these interfering substances were present. The final conclusion of the Panel was that this method, although showing distinct promise, was not capable of general application until a more sensitive fluorimeter giving greater reproducibility was available. The Panel devised a deflection instrument of the Cohen type, which provided about 4 times the sensitivity of the old Spekker Fluorimeter, but did not find this to be satisfactory. Our own investigation of this instrument shows it to be unsatisfactory for riboflavine assays because of its high instrumental blank (see Table I).

TABLE I

PERFORMANCE OF DIFFERENT FLUORIMETERS IN RIBOFLAVINE ASSAYS

Type of fluorimeter					Relative sensitivity	Instrumental blank as per cent. of F/1
Old Spekker	...	...	...	...	1	3
New ,,	...	...	...	...	7	10 to 15
Cohen type...	...	...	...	...	3 to 4	15
Electronic ...	...	...	...	...	63 to 73	1 to 2

Notes.—Relative sensitivities measured by deflections produced with F/1 using maximum sensitivity of each fluorimeter and the set-up of filters and cuvettes normally used in assays. Results given in comparison with Old Spekker as unity.
Instrumental blank determined with cuvette filled with distilled water and the usual set-up of filters.
Cohen type fluorimeter was prototype circulated to members of the Chemical Panel for critical examination, and was constructed to take test tubes instead of cuvettes.
Electronic fluorimeter used with photomultiplier at 78 volts per stage.

Attempts have been made to overcome the difficulty by developing a more sensitive fluorimeter which would permit the use of more selective filters and thus eliminate non-specific fluorescence in other parts of the spectrum. A prototype of the new Spekker fluorimeter (Hilger and

Watts) was found to possess still greater sensitivity, but its instrumental blank was still rather high. This blank has since been considerably reduced by improvements in the cuvette housing and by the use of black glass lids for the cuvettes. The improved instrument may, in our opinion, provide valuable service in riboflavine assays, especially if it is modified as we have recommended to take the new electron photomultipliers. We have been experimenting for over 2 years on the use of the latter to replace barrier layer photocells, and have constructed what is probably the most sensitive fluorimeter so far described in this country. Full details of this electronic fluorimeter were given to the Society of Public Analysts[9]. It employs as photoelectric detector either the RCA 1.P.21 or RCA 1.P.28 photo-tube or suitable British equivalents with a specially designed power pack and stable galvanometer. Table I shows that the sensitivity with the photomultiplier at 78 volts per stage is 63 to 73 times that of the old Spekker fluorimeter, and the instrumental blank has been brought quite low. It has given satisfaction during over a year's constant use, many of the results in this paper having been obtained with it.

Fluorimetric Method

All chemicals to be of Reagent or similar quality. Pyridine to be freshly redistilled.

Extraction and hydrolysis. Weigh a sample containing about 25 to 35 µg. of riboflavine. Add 50 ml. of 50 per cent. v/v concentrated hydrochloric acid and heat in a boiling water bath for 20 minutes. Cool. Make up to 100 ml. with distilled water. Filter.

Oxidation. To 30 ml. of extract adjusted to pH 4 to 4·5 with saturated solution of sodium acetate, add 1 ml. of 4 per cent. potassium permanganate solution. If the pink colour does not persist for 1 minute, add further 1 ml. quantities of permanganate solution until it does. Remove excess of permanganate by adding hydrogen peroxide (3 per cent.) drop by drop. Adjust the pH to 4·5. Add sufficient pyridine to bring the concentration of pyridine to 1 per cent.

Adsorption and elution. Prepare an adsorption column of special fuller's earth 8 to 10 cm. long.* Wash the column with about 25 ml. of 2 per cent. acetic acid solution, followed by 15 ml. of water. Pour the whole of the oxidised extract through the column. Wash the column with 1 per cent. pyridine, examining in ultra-violet light, until the yellow band due to riboflavine is clearly defined on the column. Elute with solvent (20 per cent. pyridine in 2 per cent. acetic acid), collecting the yellow fluorescent eluate. Dilute to 50 ml. with solvent.

Fluorimetry. Dilute a suitable aliquot of eluate with solvent to produce 25 ml. dilution containing about 0·15 µg riboflavine/ml. (= U). To another equal aliquot of eluate add 2 ml. of riboflavine standard (0·5 µg./ml.) in solvent and dilupte with solvent to 25 ml. (= UR). Take 5 ml. of riboflavine standard (0·5 µg./ml.) and dilute to 25 ml. (= S).

* Florisil brand 60/100 mesh (Wilkens-Anderson, Co., Chicago) has been found suitable.

Measure the fluorescence of U, UR, S and solvent only ($=$ SB) against F/4 (0·25 µg. fluorescein/ml. in phosphate buffer pH 7) separately in the same cuvette, using as primary filter Wratten 47 protected by H503 or other suitable heat-resisting filter, and as secondary filter Chance's orange OY2 or other suitable filter.

Calculation. Convert all densities into antilogarithms. Take reciprocals of antilog. densities for solutions weaker than the F/4 standard. Let the results be U, UR, S and SB. Subtract SB from U, UR and S to obtain net fluorescence for each. Then, assuming that the net fluorescence is proportional to the concentration

$$\text{concentration of riboflavine in the U dilution} = \frac{\text{(S—SB)} \times \text{concentration of riboflavine in dilution of standard}}{\text{U} - \text{SB}}$$

$$\text{concentration of riboflavine in the UR dilution} = \frac{\text{(S—SB)} \times \text{concentration of riboflavine in dilution of standard}}{\text{UR} - \text{SB}}$$

Calculate the concentration of riboflavine in the sample using the data for weight of sample taken and volume of aliquot of eluate taken. The result can be corrected for percentage recovery of the riboflavine added to UR by multiplying by $\dfrac{100}{\text{percentage recovery}}$.

Calibration of Fluorimeter. The usual method of calibrating a fluorimeter is to measure the fluorescence of a series of solutions, of which the strongest has 4 to 8 times the concentration of the weakest. If the fluorescence plotted against the concentration gives a reasonably straight line, the linearity of response is considered satisfactory over the given concentration range, which may be rather narrow. Thus in a recent paper[10] a calibration curve for riboflavine was given based only on 4 solutions ranging from 0·06 to 0·24 µg./ml.

Our experience of riboflavine assays has led us to believe that calibration should be carried out over a much wider concentration range, including especially more dilute solutions to permit accurate evaluation of the blank. Moreover, we find it preferable to evaluate the linearity of response not visually from a calibration curve, but mathematically by calculating the ratio of net fluorescence to concentration of riboflavine, which should remain constant if the response is strictly linear. Table II gives details of one of our calibration experiments with the electronic fluorimeter. Column 2 in the Table gives the mean densities of a series of riboflavine solutions ranging from 1 down to 1/32 µg./ml. (approximately 0·03 µg./ml.) as compared with fluorescein standard F/4. Column 3 gives the antilogs. of these densities and Column 4 the reciprocals of these antilogs. for the more dilute riboflavine solutions of which the fluorescence was weaker than that of the fluorescein standard, so that the zero was set on the riboflavine. Column 5 gives the *gross* fluorescence of each riboflavine solution as percentage of that of the fluorescein standard, and Column 6 gives the corresponding *net* fluorescence, calculated by deducting from the gross fluorescence the

fluorescence of the solvent only in the same cuvette (" solvent blank "). Column 7 gives the ratio of the net fluorescence to the concentration, and should be constant. Column 8 shows the percentage deviation of this F/C ratio from the average for the 6 riboflavine solutions. The

TABLE II

LINEARITY OF RESPONSE OF SPEKKER FLUORIMETER USING R.C.A.1.P.28 ELECTRON PHOTOMULTIPLIER

Calibration with riboflavine solutions at pH 5·7 matched against fluorescein standard F/4 ($\frac{1}{4}$ μg./ml.) using Wratten 39 and H503 primary filters and Chance's orange OY2 secondary filter.

Concentration of riboflavine μg./ml.	Mean density against F/4	Antilog. density (AD)	$\dfrac{1}{AD}$	Fluorescence as percentage of F/4	Net F	$\dfrac{F}{C}$	Percentage deviation of F/C from mean
1	2	3	4	5	6	7	8
Fluorescin at zero :—							
1	·387	2·438	—	243·8	237	237	0·2
$\frac{1}{2}$	·049	1·258	—	125·8	119	238	0·7
Riboflavine at zero:—							
$\frac{1}{4}$	·188	1·542	·649	64·9	58·2	233	1·5
$\frac{1}{8}$	·436	2·729	·366	36·6	29·9	239	1·1
1/16	·665	4·624	·216	21·6	14·9	238	0·7
1/32	·855	7·161	·140	14·0	7·3	234	1·1
Solvent blank ...	1·175	14·96	·067	6·7	—	—	—

Net F calculated by deducting solvent blank from gross fluorescence.

greatest deviation is 1·5 per cent. and the mean deviation 0·9 per cent. In further calibration experiments the mean deviation was 0·4 and 0·2 per cent. (see Table III). This compares favourably with a mean devia-

TABLE III

EFFECT OF PRIMARY FILTERS ON DEGREE OF LINEARITY, SENSITIVITY AND INSTRUMENTAL BLANK OF ELECTRONIC FLUORIMETER

Primary filter	Relative sensitivity as F/C ratio	Solvent blank as percentage of F/4	$\dfrac{Sensitivity}{solvent\ blank}$	Average percentage deviaion from linearity
Woods	6·67	18·3	0·37	0·4
Wratten 39	2·37	6·7	0·35	0·7
„ 47	1·82	3·6	0·51	0·2

tion of 0·7 per cent. in the calibration data for 4 fluorescein solutions ranging from 1·25 to 6 μg./ml. published by Cohen[11] in 1935, and not unsatisfactorily with the mean deviation of about 0·2 per cent. in data published on 5 fluorescein solutions by Umberger and La Mer[12] in 1945, especially considering that fluorescein solutions are much more stable than riboflavine solutions.

Choice of primary and secondary filters. Table III also shows the effect of using different primary filters, and thus varying the nature of the incident light beam, with the object of reducing the magnitude of the solvent blank, which influences the deviation from linearity. When the Wood's glass filter supplied with Spekker fluorimeters was replaced

by a Wratten 39 filter (placed behind the H503 heat-resisting filter used with the Wood's) the solvent blank was reduced by about two-thirds, but the relative sensitivity was also reduced in similar degree, so that the sensitivity/solvent blank ratio was scarcely altered. The mean percentage deviation from linearity was increased from 0·4 to 0·7, so there did not appear to be any advantage in using the Wratten 39 as primary filter. With the Wratten 47 filter the decrease in the solvent blank was greater than the decrease in sensitivity, the sensitivity/solvent blank ratio being raised to 0·51. Moreover, the mean percentage deviation from linearity was reduced to the low figure of 0·2. Further calibration experiments with pure riboflavine solutions confirmed that the Wratten 47 filter gave better results than Wood's glass, and did not show the Wratten 47A filter to possess any distinct advantage over the Wratten 47. This justifies our choice of the Wratten 47 as primary filter. Figure 1 compares the transmission curves of these filters with the absorption and fluorescence spectra of riboflavine. If the fluorescence of riboflavine were due mainly to light absorbed above 400 mμ, as previous evidence[3] suggests, then the Wratten 39 or 47 filters should give higher results than the Wood's glass filter, which transmits practically nothing between 400 and 700 mμ. In fact, however, the Wood's glass gives similar results to the Wratten 39 or 47 as primary filter, suggesting that some of the fluorescence may be due to light absorbed below 400 mμ. (We have encountered some variation between different samples of filters. The data given in Figure 1 were obtained on the actual filters used in our assays.)
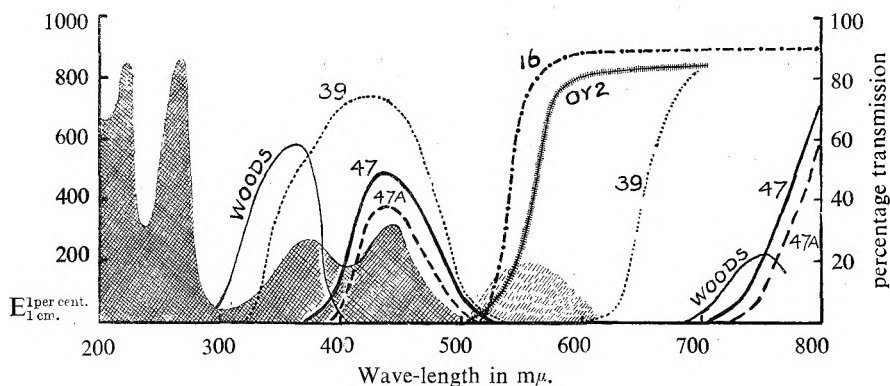
FIG. 1. Transmission curves of primary (Wood's, Wratten 39, 47, 47A) and secondary (Wratten 16, Chance's OY2) filters used in riboflavine fluorimetric assays, as compared with absorption and fluorescence spectra of the vitamin. The heavily shaded area between 200 and 500 mμ represents absorption spectrum under the given experimental conditions, and the lightly shaded area between 500 and 620 mμ indicates approximate position of fluorescence spectrum with maximum at about 570 mμ.

Turning to secondary filters, these should transmit radiation corresponding to the fluorescence spectrum of riboflavine but exclude other radiation, either from non-specific fluorescence or from exciting light reflected from the lid and sides of the cuvette[13]. Until the fluorescence

spectra of the interfering substances have been determined it is not pos-
sible to give a completely satisfactory theoretical basis for the selection
of the secondary filters to be used in assays on malted preparations.
However, since these preparations can exhibit marked non-specific blue
fluorescence, it seems desirable to use a secondary filter which does not
transmit blue light. Theoretically this requirement is fulfilled either by
the Chance's OY2 orange filter, which we have mainly used during
the last 4 years, or by the Wratten 16 which Kodicek and Wang recently
recommended. In practice the exclusion of the blue fluorescence is not
complete. For exclusion of reflected exciting light either the OY2 or
the Wratten 16 filter is unsuitable for use with the Wratten 39 as primary
filter, since the latter lets through light between 600 and 700 mμ, which
is also transmitted by these secondary filters. This disadvantage can
be overcome by using Wratten 47 or 47A as primary filter. The com-
binations of 47 primary with OY2 secondary, and of 47A primary with
16 secondary filters seem on a theoretical basis to be the best so far
devised, and have given in our hands as good analytical results as any
other combination of British or American filters we have tested, includ-
ing the Lumetron 575. However, even the best filters may not provide
effective correction when there is a considerable amount of interfering
substance present. Table IV shows the effect of using different filter

TABLE IV

EFFECT OF DIFFERENT FILTER COMBINATIONS ON INTERFERENCE OF NON-SPECIFIC BLUE
FLUORESCENCE IN MALTED PREPARATIONS

Filters				Net deflections (cm) given by		Percentage interference of blue fluorescence
Primary			Secondary	Blue non-specific	Riboflavine	
Wood's	...	...	OY2	3·35	14·7	23
39	...	...	,,	17·2	72·4	24
47	...	...	,,	5·7	26·3	22
47A	...	...	,,	2·6	11·3	23
Wood's	...	...	16	4·9	22·5	22
39	...	...	,,	25·0	127·0	28
47	...	...	,,	8·2	43·0	19
47A	...	...	,,	3·5	18·1	19
47A cal.	...	...	,,	3·4	15·9	21

Note.—Each of the above primary filters was protected by H503 filter, except the 47A Cal. where the
H503 was replaced by a Calorex heat resisting filter. The numbers of filters refer to Wratten gelatin
filters, except OY2 which is a Chance's glass filter.

combinations on a mixture of riboflavine with some of the non-specific
blue fluorescent material obtained from malted preparations as described
below. Using OY2 as secondary filter, no appreciable improvement in
results was obtained by replacing the Wood's glass by 39, 47 or 47A
primary filters. When Wratten 16 was used as secondary filter, the use
of Wratten 39 as primary filter led to less satisfactory results.

Destruction of riboflavine to provide blanks. Various procedures have

been tried for destroying the riboflavine in extracts, assuming that the residual fluorescence correctly represents the non-specific fluorescence of the extracts. We have found the following objections to these procedures : —

(a) *Sodium hydrosulphite* destroys the non-specific fluorescence as well as that of riboflavine. Whilst the latter is more readily destroyed, attempts to control the destructive process by estimating the fluorescence at different stages are baulked by the fact that sodium hydrosulphite absorbs some of the rays needed to produce fluorescence in riboflavine[10].

(b) *Irradiation* under carefully controlled conditions will destroy ribo-flavine[3], but may produce lumiflavine, the blue fluorescence of which can interfere with the fluorimetric measurements. The method is also very tedious.

(c) *Strong alkali* (*p*H above 11) will destroy riboflavine in absence of light, but the shift in *p*H reduces the fluorescence of any undestroyed riboflavine and makes it difficult to follow and control the destructive process, which otherwise can destroy non-specific fluorescence.

(d) *Stannous chloride* destroys not only riboflavine, but also non-specific fluorescence.

Removal of interfering substances. The alternative to removal of riboflavine is the removal of the interfering substances. Attempts have been made to effect this in various ways with the following results : —

(a) *Decolorisation with a fixed proportion of potassium permanganate,* followed by bleaching with peroxide, has been recommended by some workers. We found that this often led to high results, especially with malted preparations. Table V summarises some of our results on the use of permanganate and of different filters to correct for the presence of inter-

TABLE V

FLUORIMETRIC ESTIMATION OF RIBOFLAVINE IN MALT EXTRACT—CORRECTION FOR INTERFERING SUBSTANCES BY PERMANGANATE TREATMENT AND BY USE OF FILTERS MEAN FLUORIMETRIC RESULTS STATED AS PERCENTAGE OF MEAN MICROBIOLOGICAL RESULTS

Filters		New malt extract		Old malt extract	
Primary	Secondary	No Potassium permanganate	Potassium permanganate	No Potassium permanganate	Potassium permanganate
Woods	OY2	668	310	1317	502
Woods + HR ...	OY2	740	307	1566	474
Wratten 47 + HR...	OY2	496	114	1094	199
Wratten 47A + HR	OY2	570	203	1223	184
Wratten 47A + HR	Wratten 16	568	109	1144	183

Notes.—" New " malt extract was about 6 months old ; " Old " malt extract about 3 years old. Both had been stored at room temperature.   HR filter = Chance's H503.   OY2 = Chance's orange No. 3. The extracts were all made with N/10 hydrochloric acid for ½ hour in boiling water-bath. No Florisil treatment was used.

fering substances. It shows that in the absence of permanganate treatment the fluorimetric results were all much too high, and were very little affected by the use of different filters. When permanganate was used the results were all much lower. On a fairly new malt extract they were still too high with the usual set-up of Wood's and OY2 filters, but were brought much closer to the microbiological result by using Wratten 47 or 47A as primary filter. On a 3-year-old malt extract containing much more of the interfering substances the Wood's plus OY2 filters gave much too high results, and the use of the heat-resisting HR filter made no appreciable difference, showing that there was no interference from infra-red rays. Again, the use of Wratten 47 or 47A filters brought the results lower, but they were still considerably higher than the microbiological results and we had to resort to Florisil treatment to obtain satisfactory agreement. The combination of filters recommended by Kodicek and Wang was of similar efficiency to the combination we had been using. When we applied the full method of these workers, including their filters, we found that the preliminary washing of the extract with chloroform did not make the results satisfactory. As we had found the interfering blue fluorescence of malted preparations by no means completely soluble in chloroform, we were not surprised at the failure of the chloroform treatment to provide complete correction. On following Kodicek and Wang's method as closely as possible we found that on our malted preparations it gave rather too high a pH (about 5) for optimal decolorisation. A pH of about 4 gives better results.

(b) *Effect of varying amount of permanganate used*. Permanganate has been used by many previous workers to eliminate interfering substances in riboflavine assays. However, none of these seems to have provided information about the effect of varying the amount of permanganate used. Most workers seem to rely on using a fixed proportion. Kodicek and Wang[10] have recognised the need for varying the amount of permanganate with different materials, but we have found their dropwise addition of permanganate very tedious with our malted preparations. Table VI gives some of our results showing that, whereas a dried yeast

TABLE VI

EFFECT OF VARYING AMOUNT OF PERMANGANATE IN RIBOFLAVINE ASSAYS USING DIFFERENT PRIMARY FILTERS

MEAN FLUORIMETRIC RESULTS STATED AS PERCENTAGE OF MEAN MICROBIOLOGICAL RESULTS

Volume of potassium permanganate solution added to aliquot ml.	Primary filter used		
	Woods	Wratten 39	Wratten 47
Yeast sample—			
0 ...	397	177	169
1 ...	116	102	92
3 ...	59	54	56
Malted food—			
3 ...	165	150	131
9 ...	98	96	103

sample needed only 1 ml. of the 4 per cent. permanganate per aliquot to give satisfactory results, and 3 ml. was far too much, a malted food sample needed 9 ml. per aliquot. Some of our old malt extracts required much more than 9 ml. Table VI shows that when too little permanganate has been used, the results can be considerably improved by using more specific filters to eliminate interfering fluorescence. The results in the Table were obtained using Florisil to remove interfering substances as described in our method above and show that it is equally essential to use the right amount of permanganate. If this is done, the choice of more selective filter combinations becomes less important.

(c) *Phase separation.* Our aqueous extracts were shaken with various immiscible organic solvents under the ultra-violet lamp to detect whether separation of the riboflavine fluorescence from non-specific fluorescence was being effected. With amyl alcohol, chloroform, ether and light petroleum, slight separation occurred, but could not be made complete. With benzene, diacetone alcohol and isobutyl alcohol no separation was detected.

(d) *Chromatographic separation.* Elvidge[2], using the Connor and Straub[14] method, examined a number of grades of fuller's earth and selected P.A. of the Fuller's Earth Union as the most suitable. We tested a sample of this grade, but did not find it to give satisfactory purification of our materials. Other samples of fuller's earth, also of alumina and of Decalso, likewise proved unsatisfactory. We then tried Florisil, which has been largely employed by American workers, and found that it removed some, but not all, of the non-specific fluorescence when used as they recommend[15]. Observations under the ultra-violet lamp showed that riboflavine is adsorbed more strongly than the interfering substances. After numerous trials we found that elution with 1 per cent. aqueous pyridine could separate most of the interfering substances, the procedure being controlled under the lamp. 2 per cent. pyridine was seen to elute riboflavine almost as rapidly as it eluted the interfering substances, so that complete separation could not be effected. We found that the adsorption of riboflavine on Florisil was equally effective between pH 1 and pH 6. Contrary to American workers[15], we did not find the adsorbed riboflavine to be removed by washing the column with large volumes of water. Elution with 20 per cent. pyridine in 2 per cent. acetic acid gave recoveries ranging from 90 to 100 per cent.

Riboflavine in malt extract. On studying the history of the 5 malt extracts for which fluorimetric and microbiological results were given in our paper[1], we found that the extract on which the fluorimetric method gave a much higher result than the microbiological method was in fact an old one. This led us to compare the fluorimetric and microbiological results on a series of malt extracts of different known ages, all stored at room temperature. The results plotted in Figure 2 showed a definite tendency for the fluorimetric/microbiological ratio to increase as the extracts become older. Assuming that a difference of 20 per

cent. between the two methods was significant, then in 8 extracts less than 2 months old, not one showed a significant difference between the two methods, in 8 extracts 40 to 130 weeks old, 7 gave a fluorimetric result significantly higher than the microbiological result, the average fluorimetric/microbiological ratio being 1·78 and in 7 extracts $3\frac{1}{2}$ to $4\frac{1}{2}$ years old the fluorimetric result was always significantly higher than the microbiological result and the average fluorimetric/microbiological ratio was 2·64. (The fluorimetric result on one of this last 7 was too high to include in Figure 2.)

We confirmed these findings by assaying fluorimetrically and micro-biologically at intervals the riboflavine in malt extracts stored under known conditions. Figure 3 gives typical data showing a steady rise in
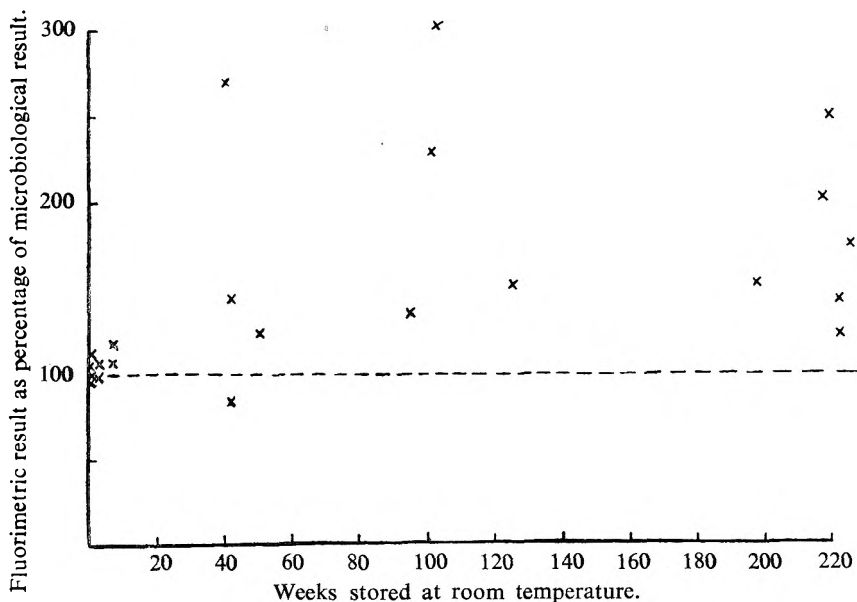
FIG. 2. Riboflavine assays on malt extract—relation between fluorimetric and microbiological results after different periods of storage.

the fluorimetric result and a steady fall in the microbiological result during $2\frac{1}{2}$ years' storage at room temperatures of a malt extract fortified with riboflavine. Similar results are being obtained with unfortified malt extract and malted preparations, but, as the riboflavine in these seems to be more stable, longer storage periods are needed for the micro-biological method to show a definite loss of the vitamin.

Non-specific fluorescence in malt extract. When using the ultra-violet lamp to control our purification procedures we discovered that old malt extracts exhibit a marked blue fluorescence which is taken up by the pyridine/acetic solvent for the riboflavine, and cannot be efficiently separated from the latter by organic solvents, e.g., benzene, chloroform, ether. This blue fluorescence is much less marked in new malt extracts. It can be developed by oxidation of the malt extract (e.g., with cold

potassium permanganate), a procedure which also develops it in aqueous extracts of barley, oats and yeast. Stronger acid extracts of these exhibit, before oxidation, a non-specific yellow fluorescence which seems not to be due to riboflavine since it is only feebly absorbed by Florisil, and is converted into a blue fluorescence by permanganate oxidation. These observations indicated that the blue fluorescence is not due to lumichrome. The spectrum of the blue fluorescence has been approximately determined using the technique described by one of us (F.W.N.)[6]. Comparison of this fluorescence spectrum and that of riboflavine with the transmission curves of various secondary filters indicated that the Wratten 47 should give the greatest degree of selectivity, but could not be expected to provide completely satisfactory correction for the non-specific fluorescence. Such correction was ensured by applying the chromatographic technique described above.
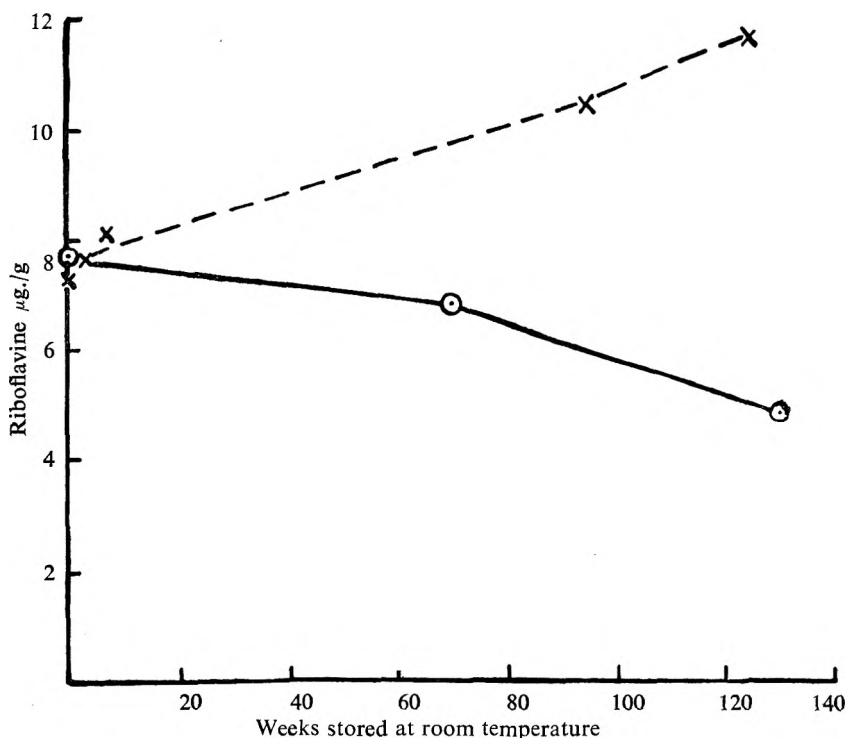
FIG. 3. Effect of storage on riboflavine content of malt extract as measured fluorimetrically X— —X and microbiologically ⊙—⊙

Effect of varying the primary filter. Our experiments with pure riboflavine solutions described in the early part of this paper showed that, as " primary filter," Wratten 47 placed behind a heat-resisting filter might be expected to give the best results. This expectation was confirmed in assays on a series of malted preparations and other foods. The average results, summarised in Table VII, showed that after adopting all the above precautions and carefully carrying out the purification

procedures, the fluorimetric results on all the samples averaged 126 per cent. of the microbiological results when using Wood's glass, 107 per cent. when using Wratten 39, and 105 per cent. when using Wratten 47. The variability in results, as indicated by the standard deviation of the mean, was also lowest with the Wratten 47 filter.

Application of improved method to old malt extracts. As a final check on our improved method we applied it, using the Wratten 47 as primary filter, to a series of old malt extracts stored under known conditions for considerable periods of time. With our previous fluorimetric method these would have given results very much higher than the microbiological results. It will be seen from Table VIII that our improved method gave on this series of old malt extracts fluorimetric results in satisfactory agreement with microbiological results, and seems to have overcome the discrepancies earlier encountered by ourselves and other workers. (The table also gives details of the fluorimetric and microbiological results

TABLE VII

EFFECT OF USING DIFFERENT PRIMARY FILTERS IN FLUORIMETRIC RIBOFLAVINE ASSAYS

Sample	Fluorimetric result as percentage of microbiological result using as primary filter behind heat-resisting filter		
	Wood's glass	Wratten 39	Wratten 47
Malt and soya food	98	97	103
Malt and soya food	174	129	122
Dried yeast	115	102	92
Flour, fortified	124	101	108
Malt yeast preparation	103	90	94
Malt food A	111	102	99
„ „ B	103	98	100
„ „ C	114	109	106
Malt extract	198	138	121
Means	126	107	105
Standard deviation of mean	10·5	5·2	3·6

TABLE VIII

FLUORIMETRIC AND MICROBIOLOGICAL ASSAYS OF RIBOFLAVINE IN MALTED PREPARATIONS AND OTHER FOODS

Sample	Riboflavine μg./g.		Fluorimetric result as percentage of microbiological
	Fluorimetric	Microbiological	
Malt extract 7½ years old	3·93	3·90	101
„ „ 7 „	4·37	4·0	109
„ „ 7 „	3·65	3·60	102
„ „ 4 „ (1)	2·5	2·4	104
„ „ 4 „	3·15	3·10	102
„ „ 3 „	4·3	4·0	108
„ „ 3 „	5·91	5·8	102
„ „ ½ „ (fortified)	14·2	15·1	94
Malted preparation (2)	11·8	11·5	103
„ „ (3)	18·5	17·1	108
„ „ (3)	23·0	23·1	100
„ „ (3)	25·0	23·8	105
Dried yeast (4)	94·1	102·0	92
Flour, fortified (4)	3·68	3·4	108

Notes.—(1) Sample prepared for the Analytical Methods Sub-Committee of the Society of Public Analysts. (2) Prepared from malted cereals and soya for clinical trials in this country. (3) Samples of baby foods. (4) Samples obtained from the Association of Vitamin Chemists, Chicago.
The average coefficient of variation for a single fluorimetric assay was 4·6 in 13 consecutive assays, and for a single microbiological assay was 5·6 in 35 consecutive assays.

on some samples mentioned in the previous table). We therefore think that the fluorimetric method we have described in this paper can be relied upon to give satisfactory results on different foods, including malted preparations, with which difficulties have previously been encountered.

DISCUSSION

We have found that in devising baby foods based on malted cereals and soya the raw materials can provide the baby's requirements of aneurine and nicotinic acid, but the content of riboflavine is more critical. By far the greater part of this vitamin in such foods comes from the malted barley, and as we have previously shown, its content of riboflavine can vary widely according to the efficiency of malting. Hence the particular need for riboflavine assays on malted products. Several years' experience with microbiological assays of riboflavine has led us to believe that these can measure the riboflavine value of a food more accurately and much more conveniently than biological assays. However, in the routine control of daily batches of food products it is not always possible to wait for the results of microbiological assays, and here fluorimetric assays are valuable.

With foods of high riboflavine content (e.g., liver, milk, yeast) good agreement with microbiological results can be provided by fluorimetric assays without resorting to chromatography for removal of interfering substances[16]. These, however, may need treatment with a reducing agent such as sodium hydrosulphite or stannous chloride. These workers then shook their extracts vigorously with air to regenerate riboflavine, but this procedure was not favoured by subsequent workers.

With foods of lower riboflavine content, including unmalted or malted cereals, more efficient purification has been found necessary. Decolorisation with permanganate, as applied to lyochromes by Koschara[17] in 1935, was employed on foods by Connor and Straub[14], who also used supersorb, a brand of fuller's earth as adsorbent, and pyridine and acetic as eluant. Amongst other adsorbents tried have been lead sulphide[18], superfiltrol[19] and Florisil[20], the two latter being special brands of fuller's earth. Several workers[4,16] have obtained satisfactory results without using adsorbents, but not on malted preparations. Difficulties in obtaining reproducible results have been reported[19]. We could not find any record of other workers using the ultra-violet lamp to check their purification procedures, and detecting the non-specific blue fluorescence we have found in malted preparations. The fact that this fluorescence may be developed by permanganate emphasises the need for caution in the use of this reagent. The low solubility of the blue fluorescent substance in chloroform show it not to be lumichrome. It is most effectively been recommended for purifying extracts[10].

SUMMARY

1. Using a more sensitive fluorimeter than has previously been described in this country, a method is given for estimating riboflavine in malted preparations which gives much better agreement with microbiological assays than had previously been obtained.

2. These preparations when oxidised with permanganate during the purification procedure exhibit a non-specific blue fluorescence which interferes with the fluorimetric assay. In old samples of malt extract this blue fluorescence is quite marked before permanganate treatment. The behaviour of the fluorescent substance with Florisil and its low solubility in chloroform show it not to be lumichrome. It is most effectively separated from riboflavine by adsorption on Florisil and careful elution with 1 per cent. pyridine, the process being observed continuously under the ultra-violet lamp.

3. Spectroscopic studies indicated Wratten 47 as a satisfactory primary filter. Comparison of fluorimetric and microbiological results confirmed this.

4. When calculating the results of riboflavine fluorimetric assays a method based on the assumption that the *net* fluorescence is proportional to the concentration, and using solvent blanks to determine the net fluorescence, is preferable to the method more usual in this country of using calibration curves obtained by plotting *gross* fluorescence against concentration.

We are indebted to Mr. E. J. Bowen, F.R.S., for advice, to Miss Janet Horsford and Mr. R. Evans for technical assistance, to Mr. G. Slaughter for the fluorescence spectrum of riboflavine, to Messrs. Hilger and Watts, Ltd., and Messrs. Kodak, Ltd., for data on the transmission of filters.

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DISCUSSION

The two papers dealing with malted preparations were discussed together.

The first was read by Dr. F. Wokes, the second by Miss C. Klatzkin.

THE CHAIRMAN said that the diastatic value introduced in the B.P., 1948, was equivalent to a Lintner value of 50, which in these days could hardly

be regarded as a moderate diastatic value. Before the war, when Canadian high diastatic malt was available, it was quite easy to obtain such values, but now with English barley, it was not possible to do so. In the Report published by the Pharmacopœia Commission before the war a standard of 15° Lintner was recommended. Unfortunately, owing to an oversight for which he must take the major responsibility, the method given in the B.P. was equivalent to a diastatic value of 50, but even a standard of 15° Lintner was not obtainable in these days with any malt, except in rare samples. The same applied to the nitrogen figure. With English malt, unless it was possible to select samples, a figure of 4·5 per cent. was quite often not attainable.

MR. G. E. SHAW (Runcorn) asked whether the term " soluble protein " was used to denote a more or less denatured protein. Had the authors any evidence for the presence of animal protein factor in a malt extract, and, if so, did it develop during gemination? The percentage of protein required in a semi-synthetic diet was very different if there was a sufficient amount of animal protein factor present. Had the authors tried paper partition chromatography for the estimation of the riboflavine?

DR. F. WOKES, in reply, said that the diastatic value of malted barley depended on the barley which was available, and Canadian barley was higher in this respect than English barley; they had had no difficulty in obtaining diastatic values and protein contents considerably higher than the minima. On the question of the solubility test for the protein, they took the American method, which was a recognised one for solid soluble protein.

He was not quite clear what was meant by the term " animal protein factor " as applied to vegetable protein. The suggestion had been made that germination might develop this so-called factor, and it was true that there did seem to be some increase in the biological value of the proteins not only of barley but of other malted cereals after they had been malted. Whether that was due to the production of an animal protein factor, or whether it was due to the destruction of inhibitors of the type of the inhibitor in soya, he did not know.

MISS C. KLATZKIN, who also replied, said that they had not done any paper partition chromatography, because they had aimed at a quantitative method. With regard to the second yellow field as well as the blue fluorescence, they had evidence in malt extract, and more particularly in yeast, of a second yellow fluorescent material which was not riboflavine.

THE CHAIRMAN asked why the authors used the B.P.C. 1923 method for diastatic value in place of the official method.

DR. F. WOKES said that they had used it in the past and had simply continued to do so. They had done more work with the B.P.C. method than with the Lintner test.

THE CHAIRMAN, in bringing the meetings to a close, said that it only remained for him to ask the members to express their thanks to those who had contributed papers to the Conference. The Science Sessions had been exceedingly well attended, and the discussions had been most valuable.

REPORT OF A SYMPOSIUM ON THE STORAGE OF DRUGS AND MEDICINES

A Symposium Session was held on Friday, September 16, 1949, at 9.30 a.m. Dr. Norman Evers, Chairman of the Conference, presided, and the opening speakers were Dr. T. E. Wallis, Mr. L. H. Boardman and Mr. J. B. Lloyd.

Dr. Wallis said that storage must be considered in relation to the various departments of pharmaceutical practice, viz.: (1) cultivation of vegetable drugs, (2) wholesale dealing, (3) hospital practice, (4) retail trade. The same commodity will often receive different treatment in the various circumstances, but in all of them the fundamental factors affecting the stored materials are the same. Special conditions arise from the nature of the premises and facilities peculiar to the different types of organisation. To fill in the details relevant to storage in the different circumstances one must, therefore, rely for information upon the experience of those pharmacists who are familiar respectively with the collecting and drying of crude drugs, with large warehouses, with the stock-rooms of hospitals, with the fitments of retail premises, or the packaging of goods for distribution.

Environment. The features of the environment which have a definite influence upon deterioration and storage are the following: —1. Atmospheric humidity; 2. Temperature; 3. Light; 4. Oxygen of the air; 5. Living agents of destruction; 6. Odorous commodities. Of all those factors, humidity and temperature appear to be the most important, since they not only have a direct and independent influence upon storage, but they also very largely govern the development of the numerous destructive living agents which abound everywhere.

Atmospheric Humidity. Moisture in the atmosphere is generally expressed in terms of humidity. When the atmosphere is completely saturated, the humidity is 100 per cent., when half saturated 50 per cent. and so on. If the humidity is over 75 per cent. it becomes dangerous in relation to storage. Under such conditions moisture is readily absorbed by certain chemical substances such as strong sulphuric acid, absolute alcohol, calcium chloride and salts of penicillin; moisture is also absorbed by some crude drugs such as squill, gelatin, gentian and digitalis. Some of the chemical substances, such as strong sulphuric acid and calcium chloride, will continue to absorb moisture until no more is present. On the other hand, when the humidity is low, water tends to be lost by some substances, such as crystalline borax, sodium carbonate and sodium phosphate, which contain a large proportion of water of crystallisation.

Moisture in the atmosphere is to some extent dependent upon the nature of the soil upon which the premises are built. A clay soil retains much moisture, whereas sandy soils lose moisture rapidly by drainage and, being non-colloidal, they do not tend to retain much moisture in loose association with the particles of the soil as usually occurs in a clay soil. Where underground cellars are used for storing stock, these considerations assume a major importance.

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Temperature. Temperature may produce effects by itself; more frequently, however, its action is associated with other features of the environment. As pointed out by Savage in 1934, absorbent cotton wool is subject to deterioration by heat alone; a raised temperature leads to a gradual loss of absorbency, due to the effect of heat in promoting a reorientation of the molecules of fatty acid present in the infinitesimal residue of cuticle left on the hairs after processing; eventually the cotton wool becomes entirely non-absorbent. For general storage, an ideal temperature is about 55° to 65°F. (10° to 14·5°C.) which, being slightly higher than the normal temperature for a great part of the year and being maintained constant day and night, reduces the humidity and minimises the risk of sudden changes of humidity. Materials so stored therefore remain dry and tendency to attack by vegetable and animal organisms is greatly reduced, because these organisms cannot exist and multiply without sufficient moisture. For certain substances which are liable at ordinary temperatures to molecular change, such as insulin, penicillin, vaccines and antibiotics generally, storage in a refrigerator is necessary. When large refrigerators are available many drugs, such as ginger and chamomile, which are specially subject to bacterial or insect attack, may be successfully stored for long periods at the low temperature provided.

Moisture and temperature together have a combined effect upon the premises and upon the materials stored in them. For example, air at 9°C. (48°F.) contained 8·7 mg. of moisture per litre, when saturated, whereas it requires twice as much moisture (viz., 17·15 mg.) to saturate one litre of air at 20°C. (68°F.). Marked changes of temperature occur with the alternation of day and night, as well as with more occasional sudden seasonal changes. If, then, there is a sudden change in temperature from 20°C. to 9°C., as when moist warm air from outside enters a cold room or house, the air becomes over-saturated and half the moisture present is thrown out in the form of water. This water is condensed upon the walls and ceilings as well as upon the contents of the room and consequently the walls stream with moisture.

Light. Exposure to sunlight will remove the colouring-matter from many drugs, especially from those which contain chlorophyll, such as leaves and herbs in general, and also from petals of flowers which contain anthocyanin pigments or coloured plastids. This loss of colour is obvious, but other changes are induced which are not visible to the eye and sensitive constituents such as the glycosides of digitalis, may be destroyed. The vitamins as a group are sensitive to light, exposure to which ultimately leads to their destruction. The active constituents of rhubarb are orange yellow in colour and gradually change under the influence of light to a pinkish tint, giving visual evidence of deterioration. Several colourless substances acquire colour by exposure to light; santonin becomes at first yellow and gradually deepens in colour till it is almost black; silver nitrate also rapidly darkens in colour and phenol gradually becomes pink.

Oxygen of the Air. Oxidation of many active principles is brought about by atmospheric oxygen, as exemplified by the resinification of

the cannabinol of Indian hemp and the gradual loss of solubility in light petroleum of the abietic acids of colophony. Linseed oil and many volatile oils, notably oils of lemon and turpentine, resinify by exposure to the air, owing to oxidation effects. Cod-liver oil contains unsaturated fatty acids which break down by the action of oxygen causing both rancidity and resinification of the oil.

Living Agents of Destruction. Moisture, temperature and oxygen together encourage the development of many living organisms which feed upon the substances stored. Moulds of various kinds are the most important vegetable organisms and animal pests include mites, silver fishes, moths, ants, small beetles and cockroaches. The most important factor in controlling all these living organisms is moisture, for without sufficient moisture protoplasm cannot retain its life and activity. A low temperature, in the neighbourhood of 0°C., is useful to prevent the development of organisms from spores and eggs, but it does not usually destroy them. The most effective means of dealing with rats and mice is to make the store proof against their entrance into it; for example, avoid wooden floors, ceilings and roofs.

Odorous Commodities. Substances such as valerian, garlic and highly perfumed soaps must be separated from one another and also from other materials in such a way as to prevent the communication of odours.

General Rules for Storage. The principal items to which attention must be directed are the following :—1. Construction of the store-room or premises; 2. Protection from dust; 3. Shelving and its arrangement; 4. Packaging; 5. Inflammable substances.

Construction of the Store-room of Premises. In general, rooms should have concrete floors and rounded corners; any crevices should be filled in with cement. Wooden plank floors should be avoided chiefly because of the number of cracks and crevices where organisms could multiply. Wooden flooring is also open to penetration by rats and mice. An equable temperature should be provided, usually cool; this is one reason why a dry cellar is often a good location for a store.

Protection from Dust. Dust contains numerous spores and small living organisms, which under favourable conditions will lead to the infestation of the stores. Dust must, therefore, be excluded as far as possible so as to avoid contamination from outside, and in this connection packing-rooms should be separate from the store. Packing materials such as straw, hay, shavings and paper should not be in or near the store; bags and sacks should also be kept outside the store. Dust also collects in the crevices and upon the ledges, grooves and guards of machinery. Machines used intermittently must not be left about in a dirty condition.

Shelving and its Arrangement. Stacks of shelving should be kept away from the walls; the lowest shelf being about 1 foot from the floor and the highest 3 feet from the ceiling. This avoids contact with condensed water streaming down walls, from moisture upon the floor and from hot moist air near the ceilings. Island stacks of shelving kept away from the walls are to be preferred to shelving actually against the walls.

Packaging and Containers. Bags and sacks should be sterilised by some process such as heating to 150°F. (65°C.) for 3 or 4 hours or by thorough washing; when filled with vegetable drugs, etc., they should be kept off the floor on a staging of slats, or they can be hung from hooks. Packages must be well closed and made of materials resistant to attack by insects and other destructive animals. Paper wrappings must be closely folded and sufficiently tightly closed to exclude moths and beetles seeking places to lay their eggs. Where the store permits access of sunlight, opaque or amber-glass containers must be used when light is deleterious to the contents.

Inflammable Substances and Poisons. Inflammable substances must be kept in a separate store well away from the main buildings. Poisons must be stored so as to comply with the regulations of the relevant legal enactments.

Control of Pests and Sterilisation of Premises. If premises or goods become infected with vegetable or animal pests, means of control and sterilisation must be adopted. It is better, however, to remember and act upon the old proverb that " prevention is better than cure."

Storage, the Pharmacopœia and the Codex. The 1948 British Pharmacopœia places increased emphasis upon the storage of drugs and chemicals, for many of which it gives instructions about storage expressed in general terms. There is also the new requirement that " vegetable drugs are required to be free from insects and other animal matter and from animal excreta." A further item to be noticed is that storage over a long period results in the deterioration of many substances having a complex molecular structure, and in certain instances, such as for some of the vaccines, the period of storage is prescribed, usually in relation also to temperature. Requirements similar to those of the British Pharmacopœia will also be appended to many of the monographs of the new British Pharmaceutical Codex.

MR. L. H. BOARDMAN said that the storage conditions laid down by the British Pharmacopœia had proved satisfactory in practice. From the manufacturer's point of view, however, storage of galenicals and pharmaceuticals covered a very wide variety of subjects.

He proposed to deal with the matter under three main headings: (1) general conditions; (2) bulk containers; (3) small containers.

1. *General Conditions.* Structure of the warehouse or factory. The drug industry includes many types of operations and utilises a great variety of basic materials, and these inherent factors within the industry should have a distinct influence upon the conditions under which drugs are stored. The most important sources of outside contamination are: (a) rodents; (b) insects, flies, cockroaches, weevils, beetles, etc.; (c) general uncleanliness, from storage bins, accumulation of dust, etc.

(a) Drugs such as cascara, liquorice, senega, senna, etc., are usually stored as received and the term " good housekeeping," so popular to-day, should be ever in the minds of those responsible for the storage of raw drugs. In modern buildings, concrete, brick and steel will usually prevent

rodents, but with older buildings every effort should be made to seal all the entries, through pipes, etc., with wire mesh, and basements should be well lighted and have no dead spaces between walls, floors, etc. A regular system of pest destruction by means of a virus preparation should be undertaken as it is practically impossible to prevent access of rodents at some time or other.

(b) Prevention of insect contamination is not easy, but cleanliness of the walls and floors and the use of a mixture of pyrethrum and D.D.T., will eliminate most insect life.

(c) Other sources of contamination are the moulds, yeasts and various bacteria, and here proper sanitation in manufacturing and packaging and proper control of the manufacturing plant and the use of clean equipment will do much to prevent contamination. For storing smaller items and powdered drugs, metal bins of stainless steel, galvanised iron or black metal, with well-fitting lids, are quite suitable and preferable to wood bins. Ergot may prove difficult to store for a lengthy period, also figs and prunes which are only allocated once a year. In these cases metal bins with tight-fitting lids and the application of chloroform have proved satisfactory; also suspending a bottle of chloroform in a bin will usually maintain these drugs in good condition. Air-conditioning and the use of ozonisers are also worthy of mention.

2. *Bulk Containers.* Galenicals may be stored in bulk containers of various materials, the chief of which are wood, stainless steel, nickel, aluminium, galvanised iron and stoneware and glass-lined steel. Wood is quite good for storing many galenicals, oak being very suitable. Casks up to 200 gallons capacity can be handled fairly easily, they stand a lot of knocking about, they can be fairly easily cleaned by treating with calcium bisulphite and hydrochloric acid followed by washing and steaming. Such containers are suitable for storage of certain liquid extracts and infusions, where the alcohol content is low. Once the cask is conditioned it can be kept in use for many years for the same galenical. Stainless steel and pure electrolytic nickel make excellent storage vessels particularly for tinctures where the alcohol content is high and everything possible must be done to minimise loss. Following some years' experience it is possible to say that with alcoholic tinctures containing 60 to 70 per cent. spirit, the loss of alcohol averages about 2 per cent. when stored in wood.

There are many varieties of stainless steel, and galenicals over long periods attack some of them; or at least the appearance, aroma and flavour of the galenical is materially altered. Preparations containing methyl salicylate slowly turn pink. Oxymel of squill darkens in colour and the flavour alters; similar remarks apply to other preparations containing acetic acid. Ammoniated tincture of quinine does not keep satisfactorily. A preparation containing ammonium carbonate and senega darkens considerably and the flavour alters. These remarks apply only to a particular alloy of stainless steel which was found quite suitable for other galenicals, e.g., camphorated tincture of opium, compound syrup of glycerophosphates and compound tincture of benzoin. The particular brand of steel must be tried out over a period of time. Pure electro-

lytic nickel is suitable for tincture of belladonna, tincture of digitalis, tincture of capsicum, tincture of nux vomica, tincture of opium, tincture of squill, tincture of ginger, liquid extract of ipecacuanha, tincture of orange, ammoniated tincture of quinine, cascara preparations and compound syrup of figs. Aluminium vessels are suitable for preparations containing methyl salicylate and for senna preparation. Stone tanks can make very useful storage vessels for large quantities of materials and acid-resisting asphalt can be used for caulking the seams. Such vessels are satisfactory for storing mildly acid preparations such as oxymel of squill and compound syrup of ferrous phosphate, and can be easily cleaned. Similarly, acid preparations can be kept in glass or earthenware, but care has to be exercised in handling. Probably the ideal containers are glass-lined metal tanks which can be used for almost any type of preparation except those which are strongly alkaline. They are easily cleaned, nevertheless they have to be handled with reasonable care, otherwise the lining chips and repairs are expensive. The initial cost is also heavy. For solid preparations such as ointments, creams and confections, stainless steel, galvanised iron or glazed earthenware is satisfactory.

Temperature of Storage. In practice a temperature of about 60°F. has proved satisfactory, but conditions can sometimes be varied with advantage, e.g., tincture of capsicum is best made and stored at a lower temperature, preferably between 30°F and 40°F. as this eliminates fatty matter which otherwise may deposit during cold weather.

Time of Storage. Most liquid galenicals tend to deposit over quite a long period, probably due to slow coagulation of colloidal matter. Senna preparations are prone to deposit over a period of many months even when filtered repeatedly. Compound syrup of glycerophosphates is another preparation which may deposit after filtration, this being considerably affected by temperature. At temperatures exceeding 90°F., calcium citrate may come down and induce other salts to come down with it. In general, galenicals should be stillaged for periods from 1 to 6 months in order to have them in the best condition. Even then different conditions in the shop can bring about changes and cause deposits.

Small Containers. In most cases glass is the substance of choice, but many other materials are being used, such as aluminium (for tablets), plastic materials (for tubes and other screw-type containers), waxed board with screw caps (for ointments and confections), and metal tubes of pure tin or tin-coated lead and aluminium. For reagent bottles the use of polyethylene is being recommended and used particularly in America. Polyethylene is said to be resistant to all acids and alkalis up to temperatures of 160°F. and many organic solvents at temperatures up to 125°F. There is, of course, much less risk from the point of view of breakage. Pure aluminium containers, preferably anodised, are also being recommended as suitable containers for concentrated hydrogen peroxide, stabilised by the addition of about 4 p.p.m. of sodium stannate or sodium pyrophosphate. Pure tin tubes are satisfactory for almost all ointments and such articles as ichthammol and emulsion base oint-

ments. A cheaper tube is made from tin-coated lead, but as the tin coating is not always evenly applied pitting may occur and corrosion set up with subsequent damage to the contents of the tube. Aluminium tubes are not always satisfactory, particularly with certain emulsion bases of the saturated fatty alcohol type. Lengthy shelf tests under varying conditions should be carried out before finally adopting tin-coated lead tubes or aluminium.

Although it may not be difficult to find a suitable container for small packs and to add a suitable preservative it is not always easy to obtain a suitable closure and liner to prevent possible chemical effect on the liner and cap and also to prevent fungoid growth on the liner due to alternate evaporation and condensation. Moulded caps of synthetic resin are better than enamelled metal closures. The latter frequently become scratched and also rust. Metal closures are, however, cheaper than plastic, and metal is still the most widely used material for machine-made caps to fit machine-made bottles. They are usually made of tin-plate or aluminium. On the advent of the plastic cap the double shell metal cap with smooth external finish was introduced. Later the " Unishell " type was introduced with a considerable saving in weight, and the design of the cap prevented the wads from falling out and to a large extent overcame the difficulty of rusting. For pharmaceutical purposes, however, the plastic cap has much to recommend it as it is generally more elegant in appearance.

The following is a list of some of the liners available with notes on their suitability for various purposes.

(a) *Ceresine.* These wads are made from paper impregnated with linseed oil and combinations of certain gums and is frequently supplied with a combination cork backing. In general, ceresine liners are not recommended for preparations with a high water content as mould growth is likely to develop, but to prevent this a waxed composition cork backing can be used, the wax containing 0·25 per cent. of nipagin T. Ceresine liners are suitable for solvents such as alcohols, benzene or turpentine.

(b) *Blackol* liners are suitable for mildly alkaline products such as milk of magnesia and emulsions and will also stand up to liquid paraffin but are not suitable for benzene or turpentine or preparations containing them. Two other different liner facings are vinglite and whiteseal which stand up to stronger acids than ceresine.

(c) *Rubber.* This is recommended for strongly alkaline products such as ammonia; it is also suitable for hypochlorites and tincture of iodine and, when suitably treated, for penicillin and its solutions. Telecothene liners have also proved very successful for tincture of iodine in tests recently carried out over a period of several months.

(d) *Tin Foil* is used for liners on products containing spirits and volatile solvents which are difficult to seal with other liners. It is also useful for cosmetic creams containing water or volatile oils.

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(e) *Alkathene.* These liners are obtainable in two forms, as the pure material or as a cork agglomerate, the latter being cork dust bound together with alkathene. This is probably the best form of wad as it has greater compressibility than pure alkathene and gives a better seal for most purposes. Alkathene itself stands up to all the strong acids and is insoluble in most solvents at room temperature. Alcohol and chlorinated hydrocarbons cause some embrittlement on exposure to bright sunlight under tropical conditions. Alkathene is resistant to caustic alkali and offers complete resistance to mould growth and attack by bacteria. Solid polythene wads are useful for volatile solvents such as nail varnish. Chemically alkathene is the same as polyethylene or polythene made by catalytic polymerisation of ethylene under pressure.

For many purposes ordinary cork has much to recommend it and waxed corks are suitable for emulsions in bulk. Pulpboard and waxed paper discs are also suitable for viscous pastes, tablets and most dry products. Many tablets are, however, packed nowadays for retail sale in special envelopes, particularly for export, using plastic film such as polythene or metal foil. Subaseal rubber caps are finding increasing use both for carboys and smaller containers, particularly for mildly alkaline products such as mixture of magnesium hydroxide. All articles should be shelf tested under varying conditions for periods of 3 to 6 months.

Sodium benzoate in 1 to 1·5 per cent. solution will prevent corrosion of many metals, and metal objects wrapped in paper or cloth containing 2 to 2·5 per cent. of sodium benzoate are protected; for example, steel, copper, brass, and soldered points; in the case of aluminium, pitting is prevented. Sodium benzoate incorporated into the adhesive prevents corrosion round the edges and underneath labels.

Liquid extract of cascara has been known to deposit after 6 months' storage at about 60°F. when the temperature is reduced by a few degrees. It is probably better to cool to about 40°F. and filter to obtain a liquid extract free from deposit. The B.P. note requires filtration after 48 hours; this is not sufficient time unless a particular temperature is specified. Liquid extract of ergot is also prone to deposit at low temperature; the British Pharmacopœia specifies that it should be kept at a low temperature and it is essential that the initial storage temperature should be low. The deposit does not appear to affect the ergotoxine content of the liquid extract. Tincture of capsicum frequently clouds and deposits at temperatures lower than 60°F. and should preferably be stored at as low a temperature as possible prior to sending out. Compound infusion of gentian may deposit after 3 months' storage when the temperature is slightly lowered; calcium tartrate has been found in the deposit and at other times crystals of hesperidin. Ethereal tincture of lobelia will also deposit a wax-like substance on exposure to cold. Simple linctus of the National Formulary may show in warm weather a semi-solid mass of crystals of invert sugar. A note regarding storage could, with advantage, be put in the N.F. Mixture of magnesium hydroxide B.P. has been found to take up both arsenic and lead when stored in certain types of bottle for periods up to 3 months.

The linctus of codeine of the National Formulary tends to ferment in hot weather, and the products of fermentation are very objectionable to taste and smell.

MR. J. B. LLOYD said that he proposed to view the subject from the standpoint of Storage in the Hospital Dispensary. In spite of the fact that the pharmacist is increasingly called upon to handle highly complex substances, many of which are inherently unstable, he thought that storage was less important than 20 years ago. The manufacturer endeavoured to produce preparations which would be stable under all possible conditions. Penicillin was perhaps the most unstable substance the pharmacist had ever been called upon to handle. For quite a time after the drug became available, it was common practice to send out injections in ice chests in order to minimise the loss of activity, but to-day it presented little or no storage problem. The material at present available could be stored at room temperature almost indefinitely; aqueous buffered injection solutions of penicillin-G could be stored at room temperature for at least 10 days, while still retaining at least 80 per cent. of their original activity. The stability of adrenaline solutions had undergone a similar improvement.

The use of preservatives and stabilising agents was not always a complete answer, as in many cases their use was, for one reason or another, undesirable. Moreover, storage often involved considerations other than the simple preservation of potency and strength. Provision must be made against contamination by dust and dirt; against chemical reaction between container and contents; against attack by insects and vermin and against reaction with atmospheric gases. Decomposition or infection by biological agencies may also have to be taken into account.

The store itself should be separate from the room in which actual dispensing was done. Adequate illumination, preferably natural, was important not only for its value in minimising mistakes due to the misreading of labels, but to keep down insect pests. Steel shelving and racking was preferable to the more usual timber. It was immune from attack by vermin, and did not provide a good foothold for climbing insects. More important, perhaps, was the fact that it is made in standard sections which may be added to at will, or transferred intact to an alternative site.

Conditions of temperature and humidity are much easier to lay down than to achieve in practice, adequate ventilation would, in general, be all that was necessary. Refrigerated storage space was, of course, required for antibiotic solutions or other biological materials. Inflammable liquids presented a serious fire risk, and statutory regulations must be observed.

Temperature was perhaps the most important factor. The higher it was the greater the velocity of chemical reaction and, within well-known limits, the growth of bacteria, moulds and yeasts. The new Pharmacopœia laid down storage conditions for quite a large range of materials.

Suitable conditions for all these substances were provided in an ordinary refrigerator. At the other end of the scale, solutions of protein hydrolysates for intravenous injection appeared to keep better at more elevated temperatures.

The Pharmacopœia was becoming increasingly concerned to specify the type of container in which substances were to be stored. Containers capable of excluding air, moisture or both were now frequently demanded, while the familiar " well-closed container " continued to be specified.

Glass was, of course, the traditional container for pharmaceuticals. Of its many advantages, not the least was that of transparency. The increasing use of parenteral solutions, however, had shown that it is not quite the inert material it was at one time thought to be, and had laid emphasis on its two principal failings, namely, its tendency to give off alkali to the contents, and the possibility of break-down with the separation of spicules. Quite recently a bottle had been submitted to him containing a solution of sodium bicarbonate which had been stored for several months, and from which a considerable quantity of glass spicules were filtered off. Had the bottle contained an opaque mixture requiring to be shaken, the consequences might have been serious. This breakdown was most apparent in containers which have undergone a heat sterilisation process, and was particularly evident in solutions of sodium citrate.

Free alkali given up to solutions from the container was provided for by an official limit test. The test, however, only applied to ampoules and similar containers of 0·5 to 25 ml. capacity. In his experience most ampoules available to-day passed the test, both on the whole ampoule and the crushed glass, but very few large containers passed when crushed. There was, in fact, no official specification for containers over 25 ml., although the United States Pharmacopœia applies a test of similar sensitivity to containers of all sizes. He suggested, therefore, that the test be extended in scope to containers of all sizes. Rubber wads, which also come into contact with the solution, varied considerably in quality, and here again an official standard would be of advantage.

Metals were rapidly coming into use as materials for containers. Distilled water stored for 12 hours in a stainless steel container had been found to have taken up lead, derived from the soldered joints. He had recently given a trial to an alloy, " Iconel," containing 80 per cent. of nickel, 14 per cent. of chromium and 6 per cent. of steel. It was more resistant to acids than monel, and seemed to offer possibilities as a bulk container for use during the preparation of large batches of sterile products. So far, he had not found any traces of heavy metals in a number of solutions stored for long periods. Like stainless steel, however, it was subject to attack by the halogens.

In these days of injections it was highly important that contamination from dust and dirt should be reduced to a minimum. From an examination of filter residues, it was apparent that many substances had

spent at least some portion of their existence in a hessian sack. For soluble substances, which were filtered during the process of preparing the injection, this did not matter a great deal, but in the case of insoluble powders it might present a serious risk. Would it not be possible for manufacturers to make a special " parenteral grade " ? Nothing more than B.P. standard of purity would normally be required, but care should be taken to see that contamination by foreign matter was reduced to the lowest possible level.

In the dispensary, dust cover stoppers should be used. Ointments presented a difficult problem, made worse by the traditional stone jar with paper cover, in which they are sent out by the wholesaler. A little reflection would bring to mind many instances in which packaging has made no progress during the last 50 years, and this in spite of the new and cheap materials which have become available. What was needed was a new approach to the problem. In the case of proprietary and branded goods, the incentives towards good packaging have produced remarkable results, but with the ordinary run of drugs, which will form the major portion of the stock of the dispensary while extemporaneous dispensing continues to exist, the position is not nearly so good. Some minor suggestions which quickly spring to mind are the packing of soft extracts, storax, caramel, etc., in collapsible tubes; the use of stoppers containing silica gel or other dehydrating material, and a host of similar ideas which in the proprietary field have become commonplace.

The CHAIRMAN, in inviting discussion, pointed out that the subject of preservatives had been discussed a year ago. It was impossible, of course, to separate preservatives entirely from the question of storage. Contributions from younger members would be particularly welcome.

MR. H. M. HIRST (Scarborough) had distinct recollections of John Whitfield, his chief nearly fifty years ago, coming to the shop in the morning and going straight to the powdered capsicum bottle and shaking it up. He himself still did that, because otherwise a mould would soon grow on the surface. In recent years it had become difficult to keep linseed meal since now it was wanted only once in three months. Glycerin and rose water would not develop a fungoid growth if made with glycerin of borax instead of glycerin. Why was compound syrup of glycerophosphates specially liable to develop mould? Points he had learned as an apprentice were never to refill a half empty bottle of sal volatile or ammoniated solution of quinine, and always to keep spirit of nitrous ether and hydrocyanic acid in inverted bottles. In many shops it would be impossible to carry out the suggestions which the opening speakers had made. Pyrethrum and D.D.T. mixed with derris was the ideal pesticide. Virus had been recommended for dealing with mice, but for mice and rats there was nothing to beat an old potato scooped out with arsenic in it.

For silver fishes sodium fluoride was best. For wasps carbon disulphide and carbon tetrachloride were better than the dangerous cyanide.

Mr. A. W. Bull (Nottingham) agreed with Dr. Wallis that in many cases humidity associated with temperature was a potent source of deterioration. Where moisture could get in, i.e., where air could get in, with fluctuation of temperature there was a constant interchange of atmosphere over the stored material in the container, and therefore the more uniform the temperature of the warehouse or stockroom the less would be that atmospheric exchange and the better the storage conditions. Many packs which to all external appearances were perefectly sealed did in fact permit atmospheric interchange, and that might be responsible for deterioration due to chemical changes where moisture started the action, It might also be the cause of contamination by odour if the material was stored near to strongly-smelling articles. It was essential to choose the correct cap and lining disc and to apply them in an efficient manner. Certain plastic caps, particularly those of larger diameter, tended to become loose on storage more readily than metal caps of similar dimensions. Rubber might be the source of contamination with zinc. In large-scale manufacture many of the tanks were of 1000 gal. capacity and upwards. In addition to the inside of the tank all the external fittings should be thoroughly cleansed—the measuring gauges, valves, pumps and pipe-lines. It must be possible to take them to pieces in units which were easy to handle. Valve seatings should be examined regularly and replaced at intervals. Where large quantities of penicillin lozenges were produced they should be stored in a well-sealed container in an air-conditioned room and packaged under the same conditions.

Mr. R. Maxwell Savage (Barnet) said that the deterioration in surgical dressings arose from the surface chemistry of the fibre, and in particular the orientation of the fatty matter which was present. Its occurrence in any particular sample of cotton wool was almost impossible to forecast. A sample which from analysis might be expected to deteriorate quickly might remain perfectly absorbent for 7 to 10 years, while another sample which seemed to be almost identical might change in a few months. A low temperature was better for storage. This problem was more troublesome in tropical climates than in temperate climates. There was probably sufficient scientific knowledge in existence to stop the trouble altogether, and the real barrier was commercial. Once the article was properly sterilised and packed it was likely to remain sterile. These articles were apt to accumulate dust, and if that happened it was not reasonable to expect the package still to retain its sterile condition when opened by the user.

Mr J. H. Oakley (London) said that Lithcote, a plastic material, was very economical for some purposes which did not require high precision standards; it had the disadvantage of chipping readily, but with containers which were carefully handled it gave a satisfactory lining. The polythene type of plastic was easily sprayed on, and gave a satis-

factory lining. Initially it was more expensive than Lithcote, but less liable to chip. It was soft and readily scratched. Sprayed metal—a mild steel container sprayed with tin or stainless steel—had not proved quite so successful in practice as had seemed likely. Stainless steel was useful for a variety of preparations of different pH, alkaline or acid. Polish greatly influenced the non-reactiveness of the stainless steel. Many stainless steels rusted if they were not highly polished. A chemical reaction seemed to be set up which pitted the stainless steel, not only shortening the life of the vessel but also giving impurities to the products which were being mixed in the steel container. In the storage of galenicals, it was important that the preparations should be issued in chronological order. Plastic wads were not very resilient and the caps tended to become loose or did not form an effective seal.

Mr. R. W. GILLHAM (Leeds) referred to the bulk packing of ointments. Earthenware pots and paper covers were untidy and difficult to manage and his firm had recently adopted waxed card containers. These were unsuitable for some types of ointment, such as those with aqueous bases and with volatile constituents like methyl salicylate, but in general they were satisfactory for ointments with paraffin bases. Ointment of yellow mercuric oxide that had been packed in waxed card containers had been found to discolour in sunlight. The darkening was found by experiment to be due to the blue end of the spectrum. The same thing occurred even with opal glass containers and they had to go back to the old-fashioned earthenware pot. If a parchment cover was used it let the light through and the ointment became discoloured. Another curious example of the effect of light was with a batch of liquid paraffin which developed a peculiar odour and taste and a pale straw colour. Many makes of rubber stopper were unsuitable for iodine bottles. Brushes having aluminium stems were unsuitable; the aluminium very rapidly combined with the iodine. Little had been said about strong smelling drugs. Infants' and invalids' foods stored in cardboard containers should be well segregated from strong-smelling drugs. Selling goods in the correct time order was very important. This was emphasised in the training of apprentices, but qualified pharmacists were not always free from blame. Sometimes drugs were returned as unsatisfactory and credit was asked for them, but the batch number showed that they were made 6 or 7 years before. The top fermentation which sometimes occurred in syrups was very troublesome. Originally the liquid might be strong enough in sugar to prevent fermentation, but evaporation from the top layer, followed by condensation, gradually weakened the top layer and moulds developed. One remedy was to shake the bottles every day, as Mr. Hirst suggested should be done with capsicum. A not very satisfactory remedy when there were thousands of bottles.

Mr. T. D. WHITTET (London) urged manufacturers of soluble sulphonamides to issue them in brown containers. The effect of light seemed to apply to a number of drugs containing amino groups, such as tablets

containing ethylenediamine, *para*-aminosalicylic acid and some of the sulphones. Storage problems in hospitals were of two types, bulk storage and storage in wards. Mention should also be made of containers issued to patients. It was desirable to try as far as possible to get a uniform type of storage container for the wards, with adequate labelling. The out-patient dispensary frequently gave out drugs which were to be kept by the patient for a month, and sometimes longer. Generally they used waxed cardboard containers for ointments, and often for tablets. Some of the waxed cartons were not suitable for hydrous ointment and other oil-in-water preparations, and also, for many tablets, such as tablets of acetyl-β-methylcholine.

For bulk storage, mobile shelving (Rollstores) was particularly useful. It was possible to fill a whole storage room with lines of these mobile shelves as long as a gangway was left. Stainless steel metal bins on casters were very useful. They could be pushed under a section of the metal shelving, and wheeled out when wanted. It was possible to get behind them for cleaning and to prevent insects and so on. Uniformity of appearance of storage containers in the pharmacy itself was of importance. Tablet containers which resembled a book about the size of the B.P., with a space for a label, made it possible to store large quantities of tablets in a small space. They had found a series of metal trays very useful for ampoules and small proprietaries. Each tray would take a couple of boxes of a certain size of ampoule and could be labelled and put in a cupboard, where they were easily accessible. With regard to the question of standards for blood bottles, a committee had been set up three years ago and had held sporadic meetings. It was hoped to standardise ½-litre and 1-litre blood bottles, of the same height and neck dimensions, so as to have a standard bottle for any transfusion fluid. The trouble had been the glass itself.

MISS V. W. BURRELL (Pinner) said that in a great many cases discoloration on storage was due not only to adverse conditions of temperature, oxidation and moisture, but also to traces of heavy metals such as copper and iron. Ascorbic acid, aneurine hydrochloride and ethyl oleate were instances. Sometimes the connecting bends or screws used in stainless steel vessels were made of brass and not entirely stainless, and this could easily be overlooked. In solutions for injection, where the prevention of discolouration was important. Seitz pads were responsible for traces of iron and, particularly, of calcium which caused precipitation with ethanolamine oleate. Calcium and zinc from rubber stoppers caused turbidity. Polyvinyl chloride liners would withstand steaming. Polythene did not stand temperatures above 100°C.

MR. R. L. STEPHENS (Brighton) said that plastic caps made with wood flour as a filler absorbed water in high humidity and gave it off in low humidity, causing a dimensional change which might be as great as 4 per cent. and which was quite sufficient to cause loosening. In that respect, metal caps were superior and when using the latter it was necessary to

choose a pliable rubber wad which would allow for the change. Cork had been used but many alcoholic pharmaceutical preparations destroyed its resilience. Lithcote and other plastic materials had the advantage of cheapness, and although they did tend to chip easily they could be fairly readily repaired. Bakelite and Lithcote were thermosetting plastics. Polyethylene as thermoplastic material is also known as Alkathene. Telcathene is the trade name for the Telegraph Construction and Maintenace Company's grade of polyethylene. Polythene was resistant to chemicals, but its resilience was not sufficient to counteract the movement of the cap, nor to take up the irregularities round the top of the bottle. It was useful for carboy stoppers and in the form of polythene film as a liner for metal boxes. Plasticised polyvinyl chloride loose liners could be used over and over again; they would keep out vermin, and would not contaminate the contents. Unplasticised polyvinyl chloride was a rigid material which would stand a temperature of about 80°C. Containers and tubes could be made from the rigid polyvinyl chloride. Plasticised polyvinyl chloride was available as tubing and had the advantage over rubber of being compounded from relatively simple materials, i.e., a single chemical which had been polymerised and a plasticiser which one could specify. For chemical purposes the plasticiser should be dioctyl phthalate, which had very little odour and was non-toxic. The material was resistant to tincture of iodine. Rubber hydrochloride had come on the market in the form of Pliofilm. A big advantage was that rubber hydrochloride was resistant to the passage of moisture vapour, whereas the other films let moisture vapour through very readily.

He had had considerable difficulty with the deterioration of the flavour and odour of liquid paraffin emulsions. Even when stored in amber containers these gave rise to an oily, rancid flavour and a strong odour. The emulsions of the B.P. and N.F. were particularly bad.

MR. J. A. MYERS (Bradford) said that most hospitals were steam heated, and steam flies came into almost every room. Dusting with pyrethrum, derris or D.D.T. was not the complete answer. Amber glass bottles were the most suitable containers for sterile solutions of sodium citrate, and he would like to ask whether there was any white glass which was resistant to sodium citrate and did not form spicules on autoclaving. Was there a cheap canister which could be recommended for ward storage of kaolin poultice? Cotton wool in the usual packets was not suitable for export. It was amazing that it should still be stored in blue paper packets, a most unsuitable container.

MR. H. S. GRAINGER (Westminster) said that Hysil flasks were best for citrate solutions. They did not last for a great many autoclavings, but they were far better than glass bottles. Recently they had had trouble with the rubber closures used for the ordinary M.R.C. blood bottle. The rubber caps were normally treated by boiling in sodium carbonate solution for a short time and then rinsing with distilled water after being treated with a chemical detergent. The amount of permanganate

required in the B.P. test for oxidisable matter increased about tenfold after the water had been autoclaved in contact with the rubber caps and with the rubber tubing used in the drip apparatus.

MR. R. H. HENRIKSEN (London) referred to recent experience with " surplus stock " cotton wool. It was in good physical condition, but had completely lost its absorbency. In similar cases of long storage he was sure that manufacturers would be very pleased to have material returned to them.

MR. S. CANNELL (Ashton-under-Lyme) said that he could confirm that water sterilised in containers closed with rubber caps no longer passed the B.P. test. Penicillin solutions which were passed through rubber tubing, as in a drip apparatus, lost potency, and if water sterilised in rubber capped bottles was used for the preparation of penicillin solutions they might deteriorate more rapidly.

MR. E. H. REID (Dagenham) asked whether Mr. Boardman had any experience of accelerated storage tests.

DR. W. MITCHELL (London) said that the remedy for silver fish was pyrethrum as a spray or aerosol. In general, pyrethrum was extremely valuable and, if it was applied as an insecticidal fog it penetrated into the crevices, but its effect was not persistent. Some drugs had a concealed infestation. Calabar beans could look quite sound and yet be empty. The ideal plant for handling and storage was glass-lined steel or enamelled cast-iron in conjunction with glass pipe-lines. It was expensive and it was necessary to be careful about the many gaskets used in the pipe-lines. More care was necessary when using stainless steel, which was the next best thing. One had to be careful that the fabricator did not solder the seams, and that the fitters, or even the maker of the plant, did not introduce zinc or lead washers or brass screws. Lithcoting was a valuable and cheap process and did not chip so badly as some people had suggested.

DR. G. E. FOSTER (Dartford) referred to labelling . It was very difficult to stick a label on a sheet tin container so that it would not come off. They had tried many glues and pastes, but had not been able to find one which was suitable.

MISS O. B. FLETCHER (London), referring to the difficulty of citrate solutions forming glass spicules, said she had found that a successful preventive was the addition of 0·05 per cent, of citric acid to the original solution.

DR. E. F. HERSANT (Dagenham), asked over what period the loss of alcohol had been 2 per cent. It was with the larger size of wide-mouth bottles that the loosening of plastic caps was most noticeable, and he thought that it was due to changes of temperature and the difference in the coefficient of expansion between the cap and the glass bottle. That was borne out by the fact that it was mostly in the tropics that these complaints arose.

MR. V. REED (London) said that paraffin emulsions, when stored in the shop, seemed to develop mould more rapidly than in the case of the emulsion with phenolphthalein. He would like to know whether pyrethrum lost its activity when stored in ordinary conditions, as in the shop round. Some chemists stored it in paper or cardboard cartons.

DR. J. M. ROWSON (London) said that his experience of the storage of many hundreds of drugs was that by far the best container was the ground glass stoppered jar. Museum jars containing highly susceptible vegetable drugs had retained their contents in good condition for a very long time. Next to that he would put the Bakelite capped bottle as exceedingly efficient, especially for powders. Waxed and brown paper coverings were very unsatisfactory. For the storage of crude drugs in ordinary drawers, he had had tin containers made in three sizes, with the largest size there were four tins in the drawer; the next size divided that longitudinally so as to get eight tins in, and the next, divided also traversely, got sixteen in. To prevent insect infestation in, for example, belladonna and henbane he put 1 ml. of chloroform into each container. Perspex containers were useful, particularly for museum demonstration purposes. It was a method of display that the retail pharmacist could use more.

MR. SPEAKMAN (Birmingham), in connection with kaolin poultice, said that the makers of antiphlogistine used to put it in aluminium containers, and one could get aluminium containers holding about 1 lb. quite cheaply. They had found that polyvinyl chloride protected rubber satisfactorily against oils for 6 months.

The CHAIRMAN said that not much had been said about fermentation, which, especially during the hot summer, had been most troublesome. Some yeasts would grow in very high concentrations of sugar, but the general cause was that mentioned by Mr. Gillham—condensation in the upper part of the container forming a dilute solution on the top of the liquid. Fermentation, however, might not always be caused by yeast, and they had had a case recently of its being caused by a bacterium in malt extract. The gas produced might not always be carbon dioxide. Some time ago they had a case of fermentation in a preparation of malt extract and hæmoglobin which was caused by nitrogen-producing organisms. Rubber caps were made from an extraordinary variety of ingredients, the rubber content varying from about 20 per cent. up to nearly pure rubber, and the manufacturers were very secretive about what they put in.

DR. T. E. WALLIS, replying to the discussion, said that penetration of bags by dust, due to variations of temperature, was an important point. Museum cases were usually made " dustproof " (so called). They had some in the Society's Museum, and found that when central heating was introduced they became particularly inefficient. One of the main reasons for that was, he thought, that there was a much greater alteration in temperature inside the cases, which resulted in small currents of air going through exceedingly small cracks and producing a deposit of very

fine dust over everything, which was difficult to deal with. He thought that the reference by Mr. Whittet to steel shelving and steel containers, provided a very useful hint on how some pharmacists could considerably improve their storage conditions. He would associate with that the remarks of Dr. Rowson about the storage of drugs in tins as one of the best ways of keeping insect pests away from specimens. Storage of things like starch and chamomile in open drawers in shops was quite common, and caused deterioration. Dust and moisture got in and led to a good deal of spoilage of stock. The most important precaution to take against silver fish was to keep the place absolutely clean, and if they were found, empty the place out and whitewash it. That was an old-fashioned remedy, but it was quite a good one.

MR. L. H. BOARDMAN, who also replied, said that wood floors could always be covered with acid-resisting asphalt, which made a very satisfactory surface. Humidity and temperature were the most important factors. He thought that 60 per cent. humidity was a fairly reasonable figure to work to. Ozonisers were useful to eliminate smells; they had tried them recently and found them very satisfactory. They too had found that plastic caps tended to become loose more than metal caps, and he thanked Mr. Stephens for his explanation. It was no use buying a stainless steel tank for storage purposes unless it was welded with the material of which the tank was made. If they were soldered, they were not stainless steel tanks.

Some types of rubber stoppers had proved satisfactory for tincture of iodine over 4 to 6 months. They had found Telcathene very satisfactory, and better than anything else so far. He had not had any experience of spraying metals except for repairing copper pans, and he had not been very satisfied. He always asked for stainless steel containers rough polished or smooth polished, as the case might be. He was not sure that that was the answer to the problem of keeping these in good condition and helping the storage, but it might be part of the answer. Galenicals were always sent out in chronological order, and that was very important. If old stocks were found in the pharmacy he did not think that any blame could be attached to the manufacturer or wholesaler. Polythene and york dust made good liners, because they were more resilient that the polythenes themselves. They had found in the past that mercuric oxide from various makers discoloured, traces of metals probably catalysing the change. They had never found liquid paraffin to go wrong, but there was no doubt that the emulsion of the N.F. did go off very quickly, and in his opinion it had not nearly enough preservative in it. The B.P. emulsion contained two preservatives, and in a much bigger proportion than that of the N.F.

He recounted an experience of spontaneous chemical reaction in some tablets of ammonium chloride and sodium nitrate when packed in a large waxed container. He would like to thank Mr. Stephens for his excellent discourse on plastics, from which he had obtained quite a few hints. Dr. Mitchell spoke highly of pyrethrum. It did not last for long, but the

knock-down effect was tremendous. When mixed with D.D.T. he had found it perfectly satisfactory without the addition of derris, which was not pleasant to handle in bulk, particularly in powdered form. A material called Tinol was very suitable for getting labels to stick on tins. The loss of alcohol to which he had referred was 2 per cent. in 12 months. Pyrethrum lost activity on storage. Ground glass stoppered jars were very good containers for drugs, but rather expensive.

MR. J. B. LLOYD, also replying, said that by the use of the mobile type of metal shelving they had been able to increase their storage capacity about 6 times for the space available. New containers should be used in replacements for wards. A standard, or several standards, for glass containers for pharmaceuticals and foods was highly desirable. The use of acid salts had done a great deal to cut down the occurrence of glass spicules in citrate solutions. Water from a glass still with double distillation, passing through a piece of rubber tube not more than 1 foot long, had failed to satisfy the B.P. test for readily oxidisable matter. That seemed to be a general failing in distilled water which came into contact with rubber. Aneurine hydrochloride solutions were particularly liable to deterioration, and after long storage a brown precipitate might occur.

The CHAIRMAN commented on the very wide range of the subjects discussed. There were still quite a number of aspects which had hardly been mentioned. The question of tropical storage, for instance, in these days of export. This was very important to manufacturers. The symposium had been not the least successful of those which had been held up to date, and he would like to thank first of all the three opening speakers for their excellent introduction and then all those who had contributed to the discussion.

RESEARCH PAPERS

THE CHEMISTRY OF ANTI-PERNICIOUS ANÆMIA FACTORS

PART II

THE "NINHYDRIN-REACTING" HYDROLYTIC FRAGMENT OF VITAMIN B12

BY B. ELLIS, V. PETROW AND G. F. SNOOK

From the Research Laboratories, The British Drug Houses, Ltd., London, N.1

WE have previously reported[1] that hydrolysis of vitamin B_{12} with 20 per cent. hydrochloric acid at 100°C., followed by examination of the hydrolysates by unidimensional paper-strip chromatography using the technique described by Consden, Gordon and Martin[2], revealed the presence of *one* "ninhydrin-reacting" substance which could not be identified with any of the known amino-acids. Collateral studies by Brink *et al.*[3] in America, although confirming the absence of amino-acids in the hydrolysates, did not substantiate the existence of the "ninhydrin-reacting" substance. We were therefore led to re-examine and extend our observations on this fragment of the B_{12} complex and now report further data which fully supports our earlier conclusions.

Rigorous purification of crystalline samples of vitamin B_{12} failed to alter the pattern of our results. Paper chromatograms of the hydrolysates, as before, invariably revealed one spot on treatment with ninhydrin, the intensity of which, however, depended markedly on the nature of the irrigation solvent employed. Thus, pronounced purple spots were obtained with solvents consisting of, or containing, the aliphatic acids. Faint or hardly visible spots, in contrast, resulted when *n*-butyl alcohol or phenol were employed. Brink *et al.*[3], it should be added, used phenol as their irrigation solvent, a fact no doubt explaining the difference between the two sets of results.

Again, in another series of experiments the coloured moiety[1] produced by hydrolysis of vitamin B_{12} was quantitatively extracted from the diluted hydrolysate with *n*-butyl alcohol, and the colourless cobalt-free aqueous phase examined spectroscopically. Selective absorption in the ultra-violet was observed with bands and inflections at 2850, 2768, 2690, 2585 and 2500 Å (for convenience this colourless cobalt-free hydrolytic material will subsequently be referred to as "the 285 component"), similar to certain fine structure bands present in the ultra-violet obsorption spectrum of vitamin B_{12} itself. A paper chromatogram prepared from the aqueous phase, and irrigated with *iso*-butyric acid, when developed with ninhydrin, gave the typical purple spot obtained using the total hydrolysate. A second chromatogram, run parallel with the first, when examined under a low pressure mercury resonance lamp fitted with a Corning 9863 glass filter[4], showed a clearly visible localised pale-blue fluorescent area occupying a position corresponding to the "ninhydrin spot." The ultra-violet absorption of the eluate from this fluorescent area, moreover, showed selective absorption substantially the same as that of the aqueous solution from which the chromatogram was prepared.

We were thus led to believe that the "ninhydrin-reacting" substance was responsible for the fluorescence and identical with "the '285' component." In later experiments, however, when n-butyl alcohol-acetic acid was employed as the irrigation solvent for the chromatograms, an entirely different result was obtained. The control strip showed the "ninhydrin-reacting" area as before, but this area was no longer fluorescent when examined using the Corning filter. Furthermore, an eluate prepared from it was now found to be transparent to ultra-violet light, an observation which excludes an aromatic structure for the "ninhydrin-reacting" substance and leads to its formulation as an aliphatic base. The chromatogram, however, showed at least two blue fluorescent zones separated from each other and from the "ninhydrin-reacting" area. Eluates from these zones showed absorption spectra similar to each other and to "the 285 component," which had been resolved under these experimental conditions into at least two structurally related substances[5].

We have already reported our failure to identify the "ninhydrin-reacting" substance with any of the naturally occurring amino-acids[1] from which it differs in giving very pale spots with ninhydrin on chromatograms irrigated with phenol (vide supra). In addition, studies on its chromatographic behaviour led to the tentative conclusion that the compound might be slightly volatile. We therefore turned our attention to the related amino-alcohols which are known to exist in combination in many naturally occurring products (e.g., ethanolamine in gramicidin[6] and cephalin[7]; 2-aminopropanol in ergometrine[8]). The ninhydrin-reacting" substance proved to be different from ethanolamine, as separation of the two occurred on paper chromatograms irrigated with phenol and with collidine respectively. When the behaviour of 2-aminopropanol was investigated, R_F values identical with those of the "ninhydrin-reacting" substance were obtained on chromatograms irrigated respectively with iso-butyric acid, n-butanol-acetic acid and phenol (see experimental part). Slightly different results were given using collidine. Separation of a mixture of the two substances did not occur, a single, nearly circular spot being obtained with ninhydrin. 2-Aminopropanol itself, on the other hand, gave rise to an elongated zone.

Whilst these experiments were in progress we had noted, inter alia, that substantial quantities of ammonia were formed when vitamin B_{12} was heated with acid or alkali. Ammonium chloride was therefore present in acid hydrolysates of vitamin B_{12}. We therefore examined the effect of adding ammonium chloride to the 2-aminopropanol prior to paper chromatography in order to simulate the ionic environment of the "ninhydrin-reacting" substance. Irrigation with collidine, followed by development with ninhydrin, now gave a nearly circular spot identical in R_F value with the vitamin B_{12} "ninhydrin-reacting" hydrolytic fragment. As 2-aminopropanol and the "ninhydrin-reacting" substance appear to have identical partition coefficients in four different solvent systems, it seems difficult to avoid drawing the conclusion that the two substances are identical. A final decision on this point must, of course, rest on a rigid chemical comparison.

Finally, parallel hydrolyses of a sample of vitamin B_{12} (Merck) (prepared by Merck & Co., Inc., and kindly sent to us through the courtesy of Dr. Randolph T. Major) and of our product, followed by detection of the " ninhydrin-reacting " fragment on paper chromatograms irrigated with 65 per cent. iso-butyric acid and with n-butyl alcohol-acetic acid respectively, have given identical results as shown in the accompanying photograph (Fig. 1). Our previous conclusions[1] are thus reaffirmed and the presence of a " ninhydrin-reacting " fragment in hydrolysates of vitamin B_{12} firmly established.

EXPERIMENTAL

Whatman No. 1 filter paper was used for all chromatograms. Solvents for irrigation were saturated with water, with the exception of iso-butyric acid which was used as a 65 per cent. aqueous solution " Analar " Grade phenol was employed. Collidine (2 : 4 : 6-trimethyl pyridine) was purified by the method of Consden et al.[9]. Other solvents were purified by distillation. Chromatograms, after irrigation, were dried in air, sprayed with
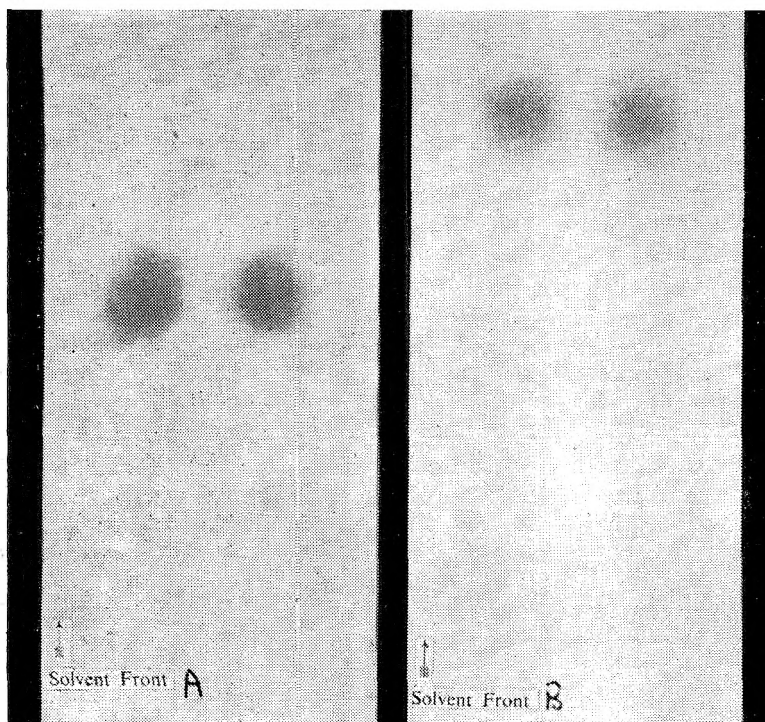
Fig. 1.—Photograph showing identity of behaviour on paper chromatograms on irrigation with (A) isobutyric acid and (B) n-butyl alcohol-acetic acid of 'ninhydrin-reacting' substances present in hydrolysates of (1) Vitamin B_{12} (Merck) (left hand spot on each paper) and (2) Vitamin B_{12} isolated by authors (right hand spot on each paper). Only the lower parts of the chromatograms are reproduced.

a freshly prepared 0·1 per cent. solution of ninhydrin in aqueous *n*-butyl alcohol and then heated for 15 minutes at 90°C. in order to develop the colours. R_F values obtained with *iso*-butyric acid, *n*-butanol, and collidine were reproducible to within 5 per cent. Greater variations were observed with phenol, and in places of R_F values the positions of spots relative to the position occupied by valine, used as a marker, are given.

Purification of crystalline vitamin B_{12}.—A sample (30 mg.) of twice recrystallised vitamin B_{12} was chromatographed on a column (1·4 cm. diam.) consisting of a mixture of aluminium silicate and kieselguhr. The sharply defined red band was developed to a distance of 12 cm. down the column, when it was dissected out, eluted, and the vitamin B_{12} recovered and recrystallised twice from aqueous acetone yielding 21 mg. of repurified crystalline product.

Paper chromatography of acid hydrolysates of vitamin B_{12}.—(1) 3 mg. of the sample of crystals prepared as described above was hydrolysed for 6 hours with 0·5 ml. of 20 per cent. hydrochloric acid in a sealed tube at 100°C. The product was evaporated to dryness *in vacuo* and the coloured residue treated with 100 microlitres of distilled water. Five microlitre quantities of this solution were spotted on to paper strips in the usual way. The strips were irrigated overnight and then developed with ninhydrin with the following results.

iso-Butyric acid.—A pronounced purple spot having R_F 0·76, was obtained superimposed upon the tail of the pigment present, which formed a pale orange-red streak extending almost to the solvent front. A sample of vitamin B_{12} (Merck), hydrolysed and chromatographed in the same way, gave an identically placed spot (paper strip A in Fig. 1).

Phenol.—The spot obtained was very weak indeed and appeared near the head of the pigment streak. It occupied a position in front of that of valine, run alongside and used as a marker. A significant change in the position of the spot did not occur when the vessel at the bottom of the chamber contained 50 per cent. acetic acid, but the intensity of the colour produced with ninhydrin was greatly increased. A weak spot was also obtained by substituting the acetic acid in the vessel by 5 N hydrochloric acid. In this case, however the " ninhydrin-reacting " substance travelled more slowly and was located a short distance behind valine, again used as a marker.

n-Butyl alcohol.—A pale spot appeared with R_F 0·20. The intensity and position was not altered by the presence of ammonia or potassium cyanide in the chamber. The pigment did not migrate.

Collidine.—A blue spot was obtained having R_F 0·34.

n-Butyl alcohol-acetic acid.—*n*-Butyl alcohol (4 vols.), acetic acid (1 vol.), and water (5 vols.) were mixed and the upper layer used as the solvent. Intense purple spots were obtained both with our sample of vitamin B_{12} and with vitamin B_{12} (Merck) as shown in Fig. 1 (paper strip B).

Caproic Acid.—In the one experiment carried out, a pronounced bluish-purple spot was obtained having R   0·53.

(2) Specimens of vitamin B_{12} were hydrolysed with 20 per cent. hydrochloric acid for (a) 5 days at room temperature and (b) 6 hours at 150°C. The " ninhydrin-reacting " substance was detected on chromatograms prepared from both these hydrolysates.

(3) 3·2 mg. of vitamin B_{12} were hydrolysed with 1 ml. of 20 per cent. hydrochloric acid by heating in a sealed tube at 100°C. for $6\frac{3}{4}$ hours. The hydrolysate was diluted to 10 ml. with distilled water and a 7 ml. portion extracted 4 times with aqueous n-butyl alcohol (3 ml. each extraction). This treatment removed the coloured moiety completely, leaving a colourless aqueous phase the ultra-violet absorption spectrum of which showed bands and inflections at 2850, 2768, 2690, 2585 and 2500 Å. Part of the solution was examined for the presence of cobalt with negative results. A portion (0·75 ml.) was taken to dryness *in vacuo*, the residue dissolved in 20 microlitres of water, and equal volumes (10 microlitres each) of this solution dispensed as two spots, 5 cm. apart, on a paper strip. After irrigation overnight with *iso*-butyric acid, the chromatogram was cut longitudinally down the centre and one half developed with ninhydrin. A purple spot appeared having R_F 0·76. Examination of the other strip under the ultra-violet light transmitted by a low-pressure mercury resonance lamp fitted with a Corning 9863 filter revealed a pale blue fluorescent spot with the same R_F value of 0·76. Black patches were not observed, thus indicating the absence of certain purines and pyrimidines.[4] The fluorescent area of the paper was cut out, eluted with 0·1 N hydrochloric acid and the ultra-violet absorption spectrum of the eluate examined. This was substantially the same as that of the aqueous phase from which the chromatogram was prepared.

Different results were obtained by the use of n-butyl alcohol-acetic acid as the irrigation solvent. In this case, development of one paper strip with ninhydrin gave rise to an intense purple spot, as already described, whilst two and possibly three fluorescent spots were revealed on the companion strip when viewed under the Corning filter. These fluorescent spots were well separated from each other and also from that area of the paper containing the substance capable of giving a colour with ninhydrin. An eluate from the latter area did not show any selective ultra-violet absorption. That a " ninhydrin-reacting " substance was, in fact, present was shown by the appearance of a typical ninhydrin spot on a chromatogram prepared from this eluate.

(4) When total hydrolysates of vitamin B_{12} were chromatographed using phenol as the irrigation solvent, the very weak spot produced with ninhydrin occupied a position in which it could easily be confused with, and even mistakenly regarded as part of, the pigment streak also present. The spot was more readily seen if the coloured moiety in a vitamin B_{12} hydrolysate was first removed by extraction with n-butyl alcohol.

Comparative behaviour of the " ninhydrin-reacting " fragment and 2-aminopropanol on paper chromatograms.—5·6 mg. of vitamin B_{12} were

hydrolysed with 20 per cent. hydrochloric acid for 6 hours in the usual way, the hydrolysate diluted, and the pigment quantitatively extracted with *n*-butyl alcohol. The product obtained on evaporation of the aqueous layer was dissolved in 250 microlitres of distilled water and this solution [solution (a)] used in the preparation of chromatograms. A 0·2 per cent. aqueous solution of 2-aminopropanol [solution (b)] was also employed.

Three spots consisting of (i) 3 microlitres of solution (a), (ii) 5 microlitres of solution (b), and (iii) a mixture of 3 microlitres of solution (a) and 5 microlitres of solution (b) were placed 2·5 cm. apart along the starting line of each paper strip (10 cm. wide and 50 cm. long). Irrigation of the strips was allowed to proceed until the solvent front had travelled in every case a distance of not less than 40 cm. The following results were obtained:

iso-*Butyric acid.* —The R_F values of the purple stops corresponding to (i) and (ii) were identical, namely, 0·76. A single spot corresponding to (iii) was obtained with the same R_F value of 0·76.

n-*Butyl alcohol -acetic acid.*—Three purple spots corresponding to (i), (ii) and (iii) were observed with identical R_F values of 0·33.

Phenol.—Acetic acid (50 per cent. v/v) was included in the chamber in order that easily visible colours should be given with ninhydrin. Valine spotted near one edge of the paper served as a marker. The apparently identical purple spots corresponding to (i), (ii) and (iii) occupied a position just in front of that of valine.

Collidine.—Both (i) and (iii) gave rise to blue spots with almost identical R_F values of *ca.* 0·32. 2-aminopropanol (ii) alone gave a blue elongated zone the limits of which extended from R_F 0·26 to R_F 0·44. The addition of several micrograms of ammonium chloride to spot (ii) prior to irrigation of the paper resulted in the appearance of a single nearly circular spot having an R_F value of 0·32.

Detection of ammonia formed on hydrolysis of vitamin B_{12}.—(a) On gently warming a mixture of 1 mg. of vitamin B_{12} and 100 microlitres of N sodium hydroxide, ammonia was evolved, detected by the appearance of a brown coloration on a piece of filter paper moistened with Nessler's reagent and held over the mixture.

(b) 10 mg. of vitamin B_{12} were hydrolysed with 0·5 ml. of 20 per cent. hydrochloric acid at 100°C. for 6 hours, the solution diluted to 10 ml., and the pigment extracted with *n*-butyl alcohol. The aqueous layer gave a colourless crystalline residue on evaporation to dryness *in vacuo.* Part of this residue was gently heated in an ignition tube with a free flame, when a white sublimate formed on the cooler upper sections of the tube. Another part of the residue, dissolved in a few microlitres of water, was mixed with one drop of cold N sodium hydroxide, and the ammonia evolved detected as described above.

SUMMARY AND CONCLUSIONS

1. Our previous conclusion that acid hydrolysis of vitamin B_{12} leads to the formation of a " ninhydrin-reacting " substance is confirmed.

2. The behaviour of this " ninhydrin-reacting " substance and of 2-aminopropanol on paper chromatograms has been examined.

3. The results appear to show that these two substances are identical.

4. Evidence has been obtained for the presence in acid hydrolysates of vitamin B_{12} of material showing selective absorption with bands and inflections at 2850, 2768, 2690, 2585 and 2500 Å (referred to as " the 285 component ").

5. This " 285 component " is resolved into at least two structurally related substances by chromatography employing n-butyl alcohol-acetic acid as the irrigation solvent.

6. Formation of ammonia occurs during acid or alkaline hydrolysis of vitamin B_{12}.

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THE CHEMISTRY OF ANTI-PERNICIOUS ANAEMIA FACTORS

PART III.   5:6-Disubstituted Benziminazoles as Products of Acid
Hydrolysis of Vitamin B_{12}

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Ellis, Petrow and Snook have shown[1] that hydrolysis of vitamin B_{12}
with 20 per cent. (w/v) hydrochloric acid at 100°C. leads to the forma
tion of a coloured cobalt-containing complex which may be quantitatively
extracted from the aqueous phase with n-butyl alcohol.   Examination of
the aqueous phase showed that, in addition to phosphate[2] and a
" ninhydrin-reacting " substance[1,3], material showing selective absorption
in the ultra-violet with bands and inflections at 2850, 2768, 2690, 2585,
and 2500 Å (" the 285-component ") was also present[3].

The presence of two main absorption band systems (2850, 2768,
2690 Å) and (2585, 2500 Å) of almost equal intensity in " the 285-
component " pointed to the existence of a dicyclic chromophore of un-
saturated or aromatic character.   The absorption spectra of aromatic
compounds of this type differ from that of " the 285-component " from
which it was concluded that a heterocyclic chromophore was present in
the latter material.   The marked fine structure of the absorption
spectrum, moreover, indicated a heterocyclic compound probably con-
taining nitrogen.   On examining the absorption spectra of a number of
heterocyclic compounds containing one or more nitrogen atoms in the
molecule, it soon became clear that ring systems containing two fused
six-membered rings could be excluded from further consideration as the
absorption invariably extended to too long a wavelength.   The study
of compounds containing a six-membered ring fused to a five-membered
ring, on the other hand, revealed the significant fact that only benzi-
minazole (III; $R = R' = H$) and indazole gave spectra resembling that of
" the 285-component," benziminazole approximating the more closely
of the two.   The characteristic long wavelength fine structure band of
benziminazole corresponding to the " 285 "-band of the B_{12} material,
however, was at much too short a wavelength, namely, $\lambda = 2730$ Å in acid
solution.

While these experiments were in progress, concurrent work briefly
referred to in Part II[3] revealed the complex character of " the 285-com-
ponent."   By employing n-butyl alcohol-acetic acid as the irrigation
solvent for the chromatograms in place of isobutyric acid which had
been used for the earlier work, resolution of " the 285-component " was
achieved.   The chromatograms, when examined under a low-pressure
mercury resonance lamp fitted with a Corning 9863 glass filter[4], now
showed three violet fluorescent zones in place of the single fluorescent
area formerly obtained and ascribed to " the 285-component."   A typical
chromatogram is represented in Figure 1 (left hand side).

```
Solvent  Front —

γ  component  —
   Rғ  O·85

β  component  —
   Rғ  O·77

α  component  —
   Rғ  O·62

                              Synthetic   5:6—
                              dimethylbenziminazole
                              Rғ   O·85

Starting  Line —
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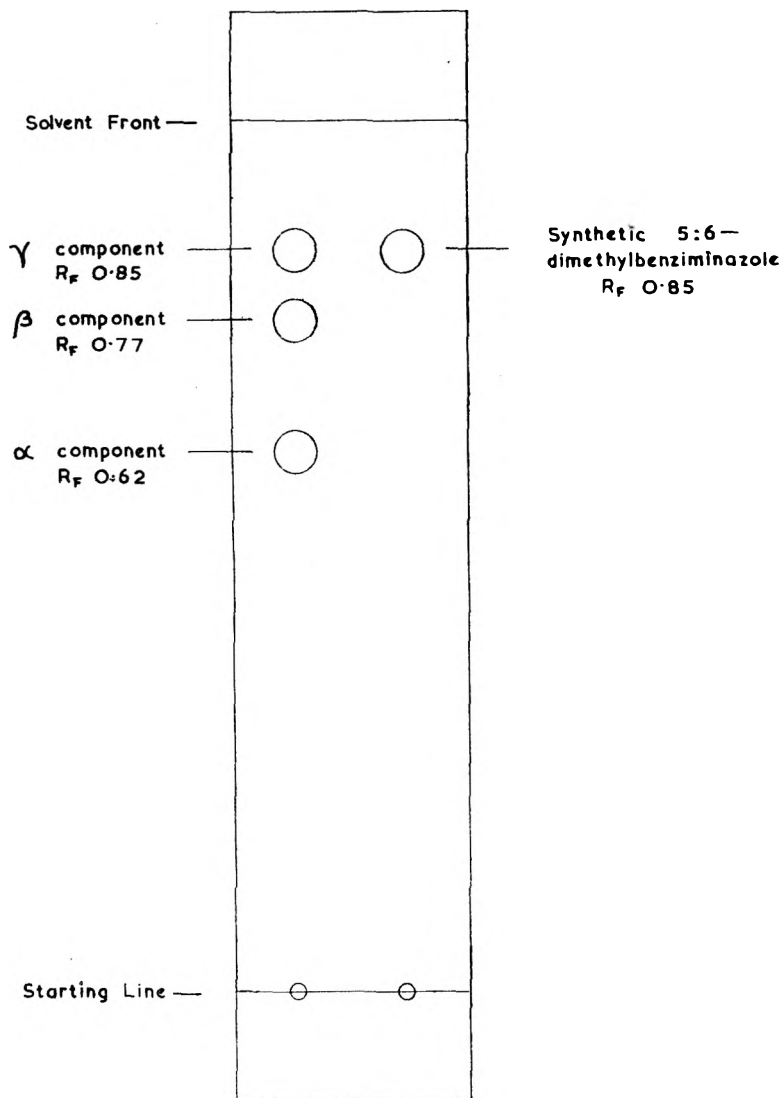
FIG. 1.—Product from vitamin B_{12} hydrolysis. Paper chromatogram irrigated with *n*-butyl alcohol-acetic acid.

Sectional elution of the different regions with dilute hydrochloric acid and spectroscopic examination of the eluates (Figure 2) showed that the compounds responsible for the three fluorescent zones had absorption characteristics similar to one another and to "the 285-component" from which they had clearly been derived. For convenience they have been designated *components* α, β, and γ. *Components* α and β, it should be added, have indistinguishable absorption characteristics which differ but slightly from those of *component* γ.
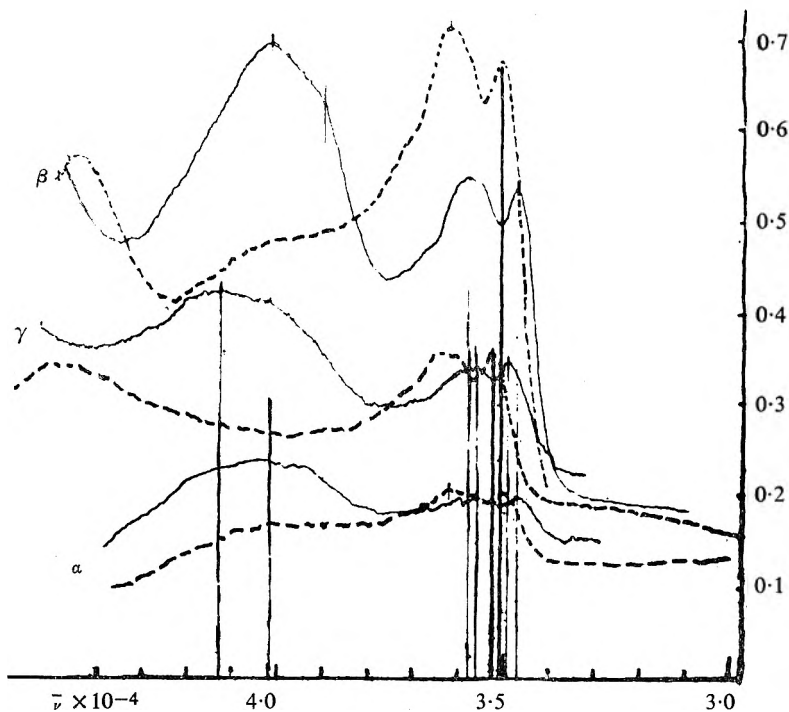
Fig. 2.—Absorption curves of eluates of fluorescent spots from a paper chromatogram of a vitamin B_{12} hydrolysate: continuous line in 0·01N sodium hydroxide, broken line in 0·01N hydrochloric acid.

The absorption spectra of *components* α, β, and γ bore a general resemblance to that of benziminazole (see Figure 3), but the characteristic long wavelength fine structure band of the latter compound still lay at a much shorter wavelength. The general form of the absorption spectrum of such a chromophore is usually little affected by substitution when the substituent groups are not conjugated to form a new or additional chromophore. The band systems are, however, shifted to a greater or lesser extent by substitution, usually in the direction of longer wavelengths.

These observations led us to conclude that *components* α, β, and γ were all substituted benziminazoles, and with the object of putting this theory to the test 22 alkylated benziminazoles were synthesised to serve as model compounds for spectroscopic measurements.

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Vitamin B$_{12}$ Hydrolysis Product ; total
aqueous extract

Benziminazole; 8·08 m.g./l.

1:5:6-Trimethylbenziminazole; 10·95 m.g./l.

5:6-Dimethylbenziminazole; 10·1 m.g./l.
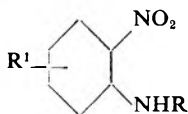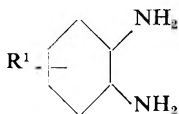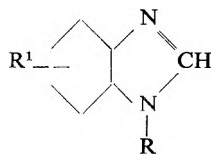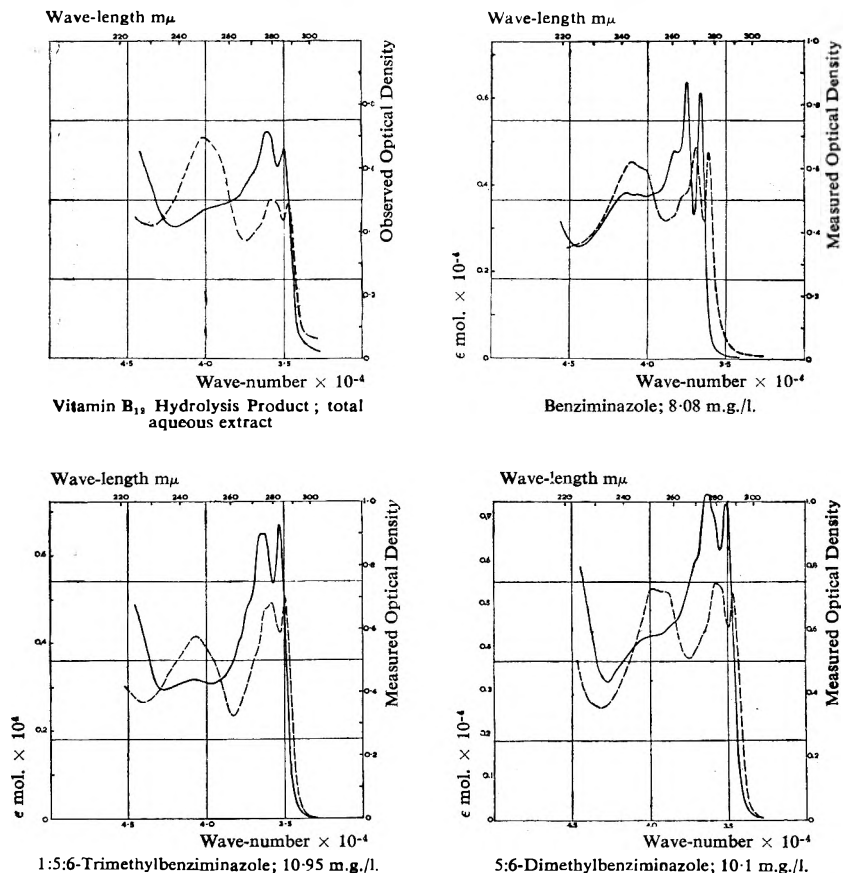
FIG. 3.—Absorption curves of benziminazoles and of the aqueous phase after *n*-butyl alcohol extraction of the vitamin B$_{12}$ hydrolysate: continuous line in 0·01N hydrochloric acid, broken line in 0·01N sodium hydroxide.

The general procedure of Phillips[5] was employed for their preparation whereby the *o*-diamine (II) was heated with formic or acetic acid in the presence of 4N hydrochloric acid. The required *o*-diamines (II) were prepared by catalytic reduction of the corresponding *o*-nitroanilines (I) employing a palladium-charcoal catalyst. N-Methyl-*o*-nitroanilines (I; R = Me) required for the preparation of the 1-methyl substituted benziminazoles (III; R = Me) were obtained by methylation of the corresponding *o*-nitroanilines (I; R = H) using the method described by Usherwood and Whiteley[6]. The preparation and properties of the following benziminazoles have not previously been reported in the literature:

1:6-, 1:7-, 2:4-, 4:5-, 5:6-*Dimethylbenziminazole.*

1:2:7-, 1:4:5-, 1:5:6-, 2:4:5-, 2:5:6-*Trimethylbenziminazole.*

1:2:4:5-, 1:2:5:6-*Tetramethylbenziminazole.*

1- and 2-monomethyl and 1:2-dimethylbenziminazole, the first benziminazoles to be synthesised and examined, showed spectra resembling that of the parent ring system, but with some alteration in

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form and small shifts in wavelength which did not approach the required values of $\lambda = 2850$ for *components* α and β, and $\lambda = 2832$ for *component* γ *(vide infra)*. A greater shift in the fine structure bands was observed with the Bz-alkylated derivatives, particularly with the 5-methyl-, 6-methyl-, 1:5-dimethyl-, and 1:6-dimethyl analogues. We therefore concentrated our synthetic efforts on the preparation of 5:6-dimethyl- and 1:5:6-trimethylbenziminazoles and, with these compounds at our disposal, were able to confirm our speculations and obtain evidence for the identity of *components* α and β with 1- substituted 5;6-dimethyl-benziminazoles and of *component* γ with 5:6-dimethylbenziminazole.

The positions of the fine-structure bands observed with these two sets of compounds in both acid and alkaline solution are recorded in Table I, and the agreements between the sets of figures is indeed seen to be remarkably good.

TABLE I

POSITION OF FINE-STRUCTURE BANDS (Å) OF HYDROLYSIS COMPONENTS OF VITAMIN B_{12}
AND OF THE CORRESPONDING METHYLATED BENZIMINAZOLES

	0·01N Hydrochloric Acid							
	a_1	a_2	a_3	a_4	a_5	b_1	b_2	
α	2850	2787	2753	2683	—	—	—	
β	2851	2787	2751	2689	2657	—	—	
1.5.6 T.M.B.	2850	2787	2751	2690	2652	2597	2470	
γ	2833	2770	2731	2673	2639	2580	—	
5.6 D.M.B.	2832	2770	2730	2672	2635	2580	2440	

	0·01N Sodium Hydroxide							
	a_1	a_2	a_3	a_4	a_5	b_1	b_2	b_3
α	2880	2812	2782	—	—	—	—	—
β	2880	2816	2782	2720	2679	2558	2492	2418
1.5 .6 T.M.B.	2880	2820	2784	2723	2685	2532	2498	—
γ	2861	2800	2760	2702	2660	2535	—	—
5.6 D.M.B.	2862	2802	2760	2705	2655	2535	2460	2380

T.M.B. = 1 : 5 : 6-Trimethylbenziminazole   D.M.B. = 5 : 6-Dimethylbenziminazole

Spectroscopic examination of the remaining alkylated benziminazoles provided further data supporting these conclusions. The difference between *components* α and β, it should be added, probably lies in the nature of the substituent groups in position 1.

All the methylated benziminazoles so far examined show characteristic spectra which are readily distinguished from each other. Each compound shows 5 or 6 bands in acid solution which are shifted in characteristic manner in alkaline solution. There are, therefore, 10 or 12 bands (12 in the case of 5:6-dimethylbenziminazole) for comparison.

As the band positions of the unknown benziminazoles agreed within the error of placing with those of the synthetic compounds, we are confident that the position of the substituents in the two sets of compounds is the same. It cannot be inferred from the identity of the spectra that the substituent groups in the vitamin B_{12} products are methyl groups. It is certain, however, that they are small unreactive groups, otherwise distortion of the spectrum to a recognisable degree could be expected. It is hardly relevant to the present communication to report the detailed spectroscopic data for all the methylated benziminazoles synthesised and examined in the course of this investigation, but it is hoped to publish this part of the work elsewhere at a later date.

Spectroscopic identification of a compound for the structure of which there is, *a priori,* no chemical evidence, is not to be undertaken lightly. Since the method is quite empirical, one is entirely dependent on the specificity of the absorption spectra for the degree of certainty with which the positive statement of identity with a model compound may be made. The spectra are so characteristic in this series of compounds, however, that they offer more strongly presumptive evidence of identity than is usually the case. In addition, preliminary experiments on the behaviour of *component* γ and 5:6-dimethylbenziminazole on paper chromatograms support the view that the two compounds are, indeed, identical.

By using the extinction coefficient of 5:6-dimethylbenziminazole as a model for reckoning molar extinctions—an assumption justified by our observation that all the benziminazoles so far examined possess very similar molar extinction coefficients—it has been possible to determine that one molecule of vitamin B_{12} gives rise to approximately one molecule of 5:6-dimethylbenziminazole (calc. as *components* $\alpha + \beta + \gamma$) on acid hydrolysis. This result accords with our preliminary analytical studies on the products of hydrolysis of vitamin B_{12}, and leads to the conclusion that *components* α, β and γ represent different stages of degradation of a common precursor.

Re-examination of the absorption spectrum of vitamin B_{12} (cf. Ellis, Petrow and Snook[1]) by the moving plate method[7] shows that two bands characteristic of benziminazoles are apparent at $\lambda = 2895$ and 2785 Å, and a trace of the third and fourth bands at $\lambda = 2630$ and 2500 Å (see Table II). Recognition of a chromophore with a complex band system such as a benziminazole is difficult, however, as the absorption due to the rest of the molecule obscures and distorts to a great extent the absorption in the benziminazole region of the spectrum. The spectrograms reproduced in Figure 4 show, however, that the moving plate method picks out the characteristic maxima and inflections. The bands are at wavelengths considerably longer than those of *components* α, β, and γ, and, in addition, the acid-alkali shift is found to be negligible (Table II, 1 and 2). These differences might be due to the nature of the group through which the benziminazole is united to the rest of the

molecule, or to intramolecular forces polarising the benziminazole chromophore. An example of the latter effect is to be found in the shift to longer wavelengths of the spectrum of the aromatic amino-acids in native proteins[8], and of purines and pyrimidines in nucleic acid[9]. In both cases mild hydrolysis releases the respective fragment from the effects of these polarising forces and the spectrum reverts to that of the free chromophore.
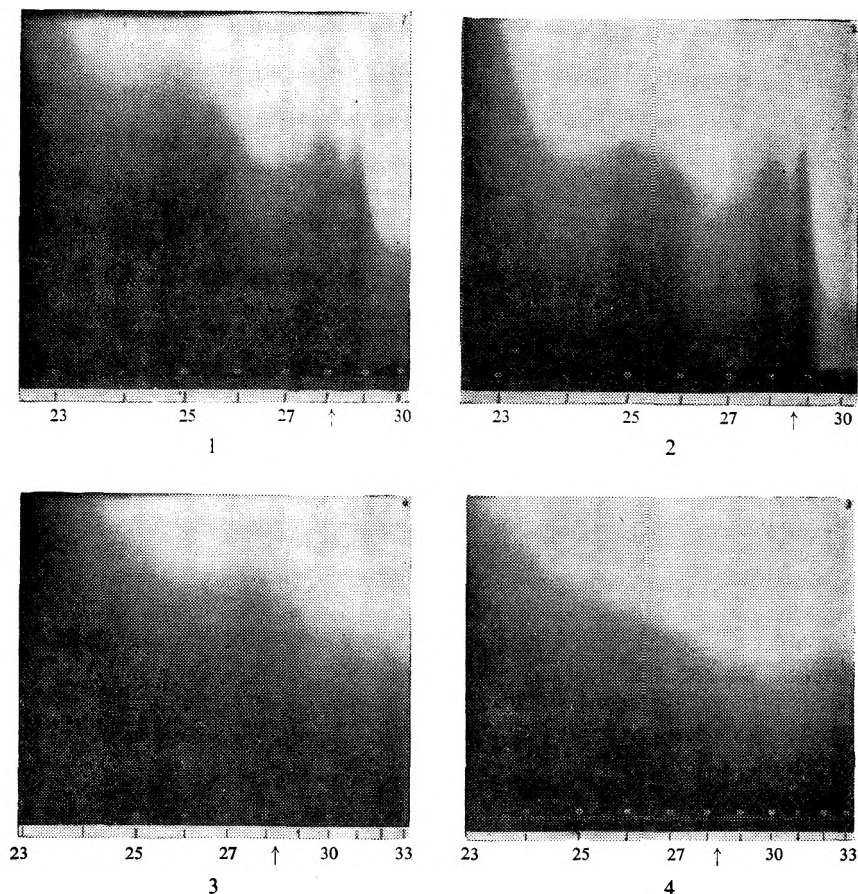
FIG. 4.—Spectrograms of :—(1) Component β; (2) 1:5:6 trimethylbenzinazole; (3) Vitamin B_{12}; (4) Methyl ester of coloured component. All measurements were made in 0·01N sodium hydroxide. The arrows mark the positions of the "285-region" bands.

A similar effect has now been observed in the case of vitamin B_{12} itself. The band positions for solutions in 3N hydrochloric acid are at shorter wavelengths than those in 0·01N hydrochloric acid solutions, and do not revert to the long-wave position on returning to the latter acid concentration. The shift to shorter wavelengths observed in 3N hydrochloric acid solution is thus irreversible. Furthermore, the new

TABLE II

POSITION OF FINE-STRUCTURE BANDS (Å) OF VITAMIN B_{12} UNDER VARYING CONDITIONS

						a_1	a_2+a_3	a_4+a_5	b_1	b_2
1	B_{12} in 0·01N sodium hydroxide	3615	3435	3235	3065	2895	2785		2630	2500
2	B_{12} in 0·01N hydrochloric acid	3610	3435	3235	3065	2895	2785			
3	B_{12} in 3N hydrochloric acid ...	3560	3415	3200	3040	2862	2770			
4	Solution (3) brought back to 0·01N hydrochloric acid ...			3210	3050	2861	2776			
5	Solution (3) brought back to weak alkali	3609	3433	3230	3070	2889	2790		2625	

Note.—Many of the bands are difficult to distinguish. Only those which have been identified with certainty are included.

positions occupied by the bands are considerably closer to those of *components* α and *β*, and these bands now show a comparable acid-alkali shift. As a moving-plate spectrogram of a solution of the " methyl ester " of the cobalt complex also present[1] in hydrolysates of vitamin B_{12} shows, apart from other changes, an almost complete absence of the fine structure bands in the " 285-region " (see Figure 4), it may be concluded that the benziminazole nucleus exists intact in the B_{12} molecule.

The recognition of a 5 : 6-dimethylbenziminazole nucleus in vitamin B_{12} raises a point of great biogenetic interest. Riboflavine, which likewise contains the 4 : 5-dimethyl-*o*-phenylenediamine residue, is known to be synthesised by microbial flora in the rumen of the sheep[10]. Tosic and Mitchell[11] have shown that the microbial flora utilise at least part of the cobalt ingested by the ruminant, and have suggested, on the basis of this observation, that pining and other wasting diseases in ruminants may be due to cobalt deficiency of the bacteria, the animal requiring not cobalt, but the bacterial products which have thereby become deficient. Becker, Smith and Loosli[12] have reported that there is no significant response in cobalt-deficient lambs when injected even with comparatively large quantities of vitamin B_{12} (125 μg.) or fed with B_{12} concentrates, although rapid disappearance of the symptoms occurred following cobalt administration by feeding (1 mg. Co per day). They concluded that these preliminary observations do not support the theory that vitamin B_{12} is an important intermediary in cobalt metabolism in lambs. Nevertheless, it seems difficult to avoid drawing the conclusion that the microbial flora of the sheep's rumen utilise cobalt to accomplish some stages in the synthesis of vitamin B_{12} or, more probably, of a closely related derivative. What part, if any, is played by riboflavine in such a process by its action on, or part in, the bacterial metabolism will, of course, only be clarified by further experimental studies.

The formation by acid hydrolysis of vitamin B_{12} of two 1-substituted 5 : 6-dimethylbenziminazoles (*components* α and *β*) leads to the conclusion that the latter ring system is attached to the macro-fragment of the B_{12} molecule by a grouping which is relatively stable to acid. The structural similarity between vitamin B_{12} (IV) and riboflavine (V) implict in

IV.

substituent
(desoxyribose residue)
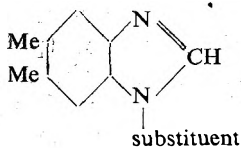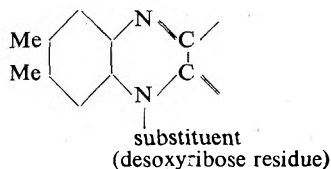
V.

the existence of an N-substituted 4:5-dimethyl-*o*-phenylenediamine residue in both compounds, may well extend to the nature of this substituent. Sugar derivatives of 5:6 dimethylbenziminazole are therefore being synthesised. Their preparation and properties will form the subject of a later communication.

<div align="center">EXPERIMENTAL</div>

M.pts. are uncorrected.

Paper Chromatography of a vitamin B_{12} hydrolysate.

1·6 mg. of vitamin B_{12} were hydrolysed with 0·5 ml. of 20 per cent. hydrochloric acid for 15 hours at 100°C., the solution diluted to 5 ml. and extracted three times with successive portions (2 ml.) of *n*-butyl alcohol. The combined *n*-butyl alcohol extracts were back extracted several times with 2 per cent. hydrochloric acid, and all the aqueous phases combined. Evaporation of these extracts to dryness gave a crystalline residue. A solution of this material in several microlitres of distilled water was spotted on to a paper strip and the latter irrigated with *n*-butyl alcohol-acetic acid. Examination of the chromatogram in $\lambda = 2536$ Å radiation revealed the presence of three violet fluorescent spots (*components* α, β, and γ) having R_F values 0·62, 0·77 and 0·85 respectively.

Chromatography of authentic 5:6-dimethylbenziminazole gave rise to a violet fluorescent spot, identical in every respect with *component* γ (see Figure 1).

Spectrographic Methods.

(i) The moving-plate method described in 1937 by one of us[7] has been modified by substituting a new cam which gives a ratio of final to initial rate of motion of the spectrograph plate of 100:1 instead of the original 10:1. This gives greater latitude and sensitivity to the method. The precision of location of fine-structure bands or inflections depends upon their spectral width and resolution. In the case of the sharper long wavelength bands of benziminazoles the error is as little as ± 1 Å, while for the diffuse short wavelength bands it may increase to ± 10 Å.

(ii) The spectrophotometric measurements were made with an automatic recording spectrophotometer designed and built by the Telecommunication Research Establishment of the Ministry of Supply in collaboration with Medical Research Council. A full description of this instrument will be published elsewhere. It plots a continuous record of optical density against wave number. Figure 2 shows examples of records taken with this instrument.

(iii) All solutions were examined in both 0·01N hydrochloric acid and sodium hydroxide. Since the change in the absorption spectrum on passing from acid to alkali is different for the different benziminazoles, the " acid " and " alkaline " spectra may be taken as independent properties of the chromophore in question and hence the specificity increased very greatly. In Figure 3 the usual notation is employed.

$$\epsilon_{mol} = \frac{1}{c \times l} \cdot D \quad \ldots\ldots\ldots\ldots (1)$$

where ϵ is the molar extinction coefficient, D is the measured optical density, l the path length and c the concentration in g.-molecules per litre.

Samples of vitamin B_{12} were dried at 70°C. for 1 hour prior to examination.

Spectrophotometry of Paper Chromatogram Eluates.

(i) The sensitivity of spectrophotometry:—The weight of substance (W_s g.) to give a satisfactory optical density (D) is related to the molar extinction coefficient (ϵ_{mol}) and the volume of the photometer cell (V ml.) for a path length of 1 cm.

In most spectrophotometers the volume of solution required to fill the cell adequately is proportional to the path length. We may, therefore, define a specific volume requirement (v_s) of the photometer as the volume required to fill a cell of 1 cm. path length. Introducing W instead of c into the relation (1) we obtain

$$W_s = \frac{v_s}{1000} \frac{DM}{\epsilon_{mol}} \quad \ldots\ldots\ldots\ldots\ldots(2)$$

where M is the molecular weight.

In our photometer 1·7 ml. is required to fill a cell of 1 cm. path length. If we take an average maximum ϵ for benziminazoles as $0·5 \times 10^4$ and a molecular weight of 150, and if we also take as a requirement that the optical density of the solution at the band maxima shall be not less than D=0·8, then it follows that the minimum weight of benziminazole required to fulfil the conditions is given by:

$$W_s = \frac{1·7 \times 0·8 \times 150}{1000 \times 0·5 \times 10^4} = 40 \ \mu g.$$

The relation (2) is general for any photometer and any compound, and is useful for rapidly determining whether the spectrophotometric method is sufficiently sensitive for estimating components of a paper chromatogram. W_s may be termed the limiting weight sensitivity of the spectrophotometer.

Where the specific absorption of a compound is high, as in the case of the benziminazoles, complete absorption curves can be derived from quantities of the order of ten micrograms.

(ii) *Identification of the components on the paper chromatogram:* Parallel runs on two spots of hydrolysate were made on the same piece

of paper. After drying the paper in air or in the oven at 95°C., the strips were examined, before cutting the paper, under the mercury resonance arc through Corning 9863 filter which transmits the $\lambda = 2536$ line (Holiday and Johnson[4]). Regions of fluorescence were observed on a typical chromatogram as described above. These were marked off with pencil, the strip cut longitudinally between the two spots, and one half sprayed with the ninhydrin reagent. The flourescent spots were cut out from the unsprayed half, together with control sections of the paper, and each eluted by soaking at least half an hour in 4 ml. 0·01N hydrochloric acid.

It may be well to emphasise that the excitation by the $\lambda = 2536$ Å radiation of the mercury arc is essential for the appearance of the fluorescent spots, which are not seen when the paper is viewed under the same arc, but through Wood's glass.

Figure 2 is a direct reproduction of the record made by the spectrophotometer on the eluates of the three fluorescent spots from such a paper chromatogram. Each pair of curves represents the same eluate in both acid and alkaline solution. In the case of each pair the spectrum shifted to longer wavelength (smaller wavenumber) is that for the alkaline solution.

Preparation of p-*Toluenesulphon-o-nitroanilides:*

The following general method was employed: The nitro-amine (0·1 mol.), dissolved in pyridine (15 to 20 ml.), was treated with *p*-toluene-sulphonylchloride (0·1 mol.) in portions. The reaction was completed by heating for 2 hours on the steam bath. After addition of dilute hydrochloric acid, the solid was collected, washed with water and purified by crystallisation. The yields were of the order 80 to 95 per cent.

p-*Toluenesulphon-(2'-nitro-4'-methylanilide)*, yellow needles from alcohol, m.pt. 104°C. Found: N, 9·2. $C_{14}H_{14}O_4N_2S$ requires N, 9·2 per cent.

p-*Toluenesulphon-(2'-nitro-6'-methylanilide,* yellow prisms from alcohol, m.pt. 125°C. Found: N, 8·8. $C_{14}H_{14}O_4N_2S$ requires N, 9·2 per cent.

p-*Toluenesulphon-(2'-nitro-3':4'-dimethylanilide)*, prismatic needles from alcohol, m.pt. 126° to 127°C. Found: N, 8·9. $C_{15}H_{16}O_4N_2S$ requires N, 8·8 per cent.

p-*Toluenesulphon-(2'-nitro-4':5'-dimethylanilide)*, yellow blades from alcohol, m.pt. 149° to 150°C. Found: N, 8·9. $C_{15}H_{16}O_4N_2S$ requires N, 8·8 per cent.

Methylation of the p-*Toluenesulphon-o-nitroanilides:* The procedure below was adopted: A mixture of the sulphonanilide (0·1 mol.) and 4N sodium hydroxide (26 ml.) was treated under reflux with methyl sulphate (8·0 ml.), the mixture being kept alkaline to phenolphthalein by drop-by-drop addition of 10 N sodium hydroxide solution. A further 8·0 ml. of methyl sulphate was added, and the mixture refluxed for 15 minutes. After cooling, the product was collected, washed with water and purified from alcohol. (Yields, 80 to 95 per cent.)

p-*Toluenesulphon-*(2'-*nitro*-N : 4'-*dimethylanilide*), pale yellow prisms, m.pt. 128° C. Found: N, 9·1. $C_{15}H_{16}O_4N_2S$ requires N, 8·8 per cent.

p-*Toluenesulphon-*(2'-*nitro*-N : 6'-*dimethylanilide*), silver leaflets, m.pt. 139° to 140°C. Found: N, 8·9. $C_{15}H_{16}O_4N_2S$ requires N, 8·8 per cent.

p-*Toluenesulphon-*(2'-*nitro*-N:3':4'-*trimethylanilide*), colourless prisms, m.pt. 137°C. Found: N, 8·7. $C_{16}H_{18}O_4N_2S$ requires N, 8·3 per cent.

p-*Toluenesulphon-*(2'-*nitro*-N : 4': 5'-*trimethylanilide*), m.pt. 125° to 127°C. Found: N, 8·3. $C_{16}H_{18}O_4N_2S$ requires N, 8·3 per cent.

Hydrolysis of the p-*Toluenesulphon-*N-*methylanilide :* A mixture of the p-toluenesulphon-N-methylanilide (0·1 mol.), glacial acetic acid (16 ml.) and concentrated sulphuric acid (37 ml.) was heated on the steam bath for 1 to 2 hours and poured into ice-water. The amine was collected and recrystallised from alcohol. Yields 60 to 75 per cent.

2-*Nitro*-N : 3 : 4-*trimethylaniline hemihydrate,* scarlet prisms, m.pt. 59° to 60°C. Found: N, 14·7. $C_9H_{12}N_2O_2.\frac{1}{2}H_2O$ requires N, 14·8 per cent. Found on material resublimed at 100°C. 0·05 mm.: N, 15·5, $C_9H_{12}N_2O_2$ requires N, 15·6 per cent.

2-*Nitro*-N : 4 : 5-*trimethylaniline* was resublimed at 100°C. 0·05 mm. for analysis, forming orange-red needles, m.pt. 138°C. Found: N, 15·6. $C_9H_{12}O_2N_2$ requires N, 15·6 per cent.

The preparation of Benziminazoles:

The appropriate nitro-amine (0·02 mol.) in ethanolic solution was shaken with hydrogen in the presence of 10 per cent. palladium-charcoal (0·5 g.) until hydrogen uptake was complete. The solution, freed from catalyst, was taken to dryness in an atmosphere of nitrogen and the residue dissolved in 4N hydrochloric acid (20 ml.). The appropriate aliphatic acid (0·1 mol.) was added and the solution refluxed in nitrogen for 40 minutes. The product was then precipitated by addition of dilute ammonia, collected and recrystallised. The yields were 50 to 60 per cent. of the theoretical.

Monomethylbenziminazoles: 1-Methyl-, needles from light petroleum, m.pt. 64°C. (Skraup[13]); 2-methyl-[5], needles from water, m.pt. 176°C.; 4-methyl-, needles from ethyl acetate-light petroleum, m.pt. 140°C. (Gabriel and Thieme[14]); 5-methyl-, needles from ethyl acetate-light petroleum, m.pt. 113°C., b.pt. 169° to 172°C./0·1 mm. (Niementowski[15]).

Dimethylbenziminazoles: 1:2-Dimethyl-, needles from ethyl acetate-light petroleum, m.pt. 109° to 110°C. (Fischer[16] gives m.pt. 112°C.); 1:5-dimethyl-, needles from ethyl acetate-light petroleum, m.pt. 94°C. (Fischer[17]); 1:6-dimethyl-, needles from light petroleum, m.pt. 74° to 75°C. Found: C, 73·7; H, 7·0. $C_9H_{10}N_2$ requires C, 73·9, H, 6·9 per cent.) (Fischer and Wreszinski[18] describe this compound as an oil, b.pt. 280°C.); 1:7-*dimethyl-,* prismatic needles from ethyl acetate-light petroleum, m.pt. 68° to 70·5°C. Found: N, 19·0. $C_9H_{10}N_2$ requires N, 19·1 per cent.; 2:4-*dimethyl-,* prisms from ethyl acetate, m.pt. 168° to 169°C. Found: C, 74·0; H, 6·6. $C_9H_{10}N_2$ requires C, 73·9; H, 6·9 per

cent. 2:5-dimethyl-, leaflets from ethyl acetate-light petroleum, m.pt. 202°C. (Green and Day[19]); 4:5-*dimethyl-,* leaflets from aqueous alcohol, m.pt. 196° to 197°C. Found: N, 18·8; $C_9H_{10}N_2$ requires N, 19·1 per cent.; 5:6-*dimethyl-,* needles from ethyl acetate-light petroleum, m.pt. 199° to 200°C. Found: C, 73·5; H, 6·4; $C_9H_{10}N_2$ requires C, 73·9; H, 6·9 per cent.

Trimethyl benziminazoles: 1:2:5-Trimethyl-, plates from ethyl acetate, m.pt. 141°C. (Fischer and Regaud[20]); 1:2:6-trimethyl-, rods from ethyl acetate-light petroleum, m.pt. 119° to 120°C. (Fischer and Rigaud[20] give m.pt. 122°C.); 1:2:7-*trimethyl-,* fine needles from petroleum, m.pt. 146° to 147°C. Found: C, 74·5; H, 7·7; $C_{10}H_{12}N_2$ requires C, 75·0; H, 7·6 per cent.; 1:4:5-*trimethyl-,* white needles from light petroleum, m.pt. 95° to 96°C. Found N, 17·7. $C_{10}H_{12}N_2$ requires N, 17·5 per cent.; 2:4:5-*trimethyl-,* needles from aqueous alcohol, m.pt. 188° to 190°C. Found: N, 171·5. $C_{10}H_{12}N_2$ requires N, 17·5 per cent.; 1:5:6-*trimethyl-,* needles from ethyl acetate-light petroleum, m.pt. 142° to 143°C. Found: N, 17·1. $C_{10}H_{12}N_2$ requires N, 17·5 per cent. 2:5:6-*trimethyl-,* needles from aqueous alcohol, m.pt. 233° to 234°C. Found: C, 75·2; H, 7·1; $C_{10}H_{12}N_2$ requires C, 75·0; H, 7·6 per cent.

Tetramethyl benziminazoles: 1:2:4:5-*Tetramethyl-,* long needles from aqueous alcohol, m.pt. 144° to 145°C. Found: N, 16·1. $C_{11}H_{14}N_2$ requires N, 16·1 per cent.; 1:2:5:6-*tetramethyl-,* pale yellow prisms from ethyl acetate-light petroleum, m.pt. 164°C. Found: N, 16·0; $C_{11}H_{14}N_2$ requires N, 16·1 per cent.

SUMMARY AND CONCLUSIONS

1. Evidence is presented for the presence of three chemically related substances, *components* α, β, and γ, in acid hydrolysates of vitamin B_{12}.

2. Spectroscopic examination of these components has led to their classification as benziminazole derivatives.

3. Spectroscopic comparison with 22 methylated benziminazoles synthesised to serve as model compounds, has resulted in the identification of *components* α and β as 1:5:6-trisubstituted benziminazoles, and of *component* γ as a 5:6-disubstituted benziminazole.

4. Preliminary paper chromatographic studies point to the identity of *component* γ with 5:6-*dimethylbenziminazole;* from which it is concluded that *components* α and β are both 1-substituted 5:6-dimethylbenziminazoles.

5. Spectroscopic and chemical evidence leads to the conclusion that only one 5:6-dimethylbenziminazole residue is released from vitamin B_{12} on acid hydrolysis and that the benziminazole nucleus exists preformed in the vitamin.

6. It is, therefore, concluded that *components* α, β, and γ represent successive stages of degradation of a common precursor.

7. The bearing of these results on the biogenesis and structure of vitamin B_{12} is briefly discussed.

It is a pleasure to acknowledge the encouragement of the Directors of The British Drug Houses, Ltd., in this work.

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SYNTHESIS OF THE BENZENE ANALOGUE OF VITAMIN A

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THE successful synthesis of pure crystalline vitamin A by Arens and van Dorp[1] and by Isler *et al.*[2] opened a new field in the chemical study of vitamin A. Thus, it is possible to synthesise various analogues of vitamin A in order to establish the relationship between chemical structure and vitamin A activity. A survey of recent publications on synthetic compounds bearing modified side chains led to the conclusion that although the terminal hydroxyl group might not be of utmost importance, the length and the general skeleton of the side chain could not be altered without a complete loss of activity. (*cf.* Table I.)

TABLE I.

Compounds		Activity (Vitamin A = 1)
R—CH : CH—C : CH—CH : CH—C : CH—COOH 　　　　｜CH$_3$　　　　　　｜CH$_3$	I. Ref. 1	1
R—CH : CH—C : CH—CH : CH—C : CH—CH$_3$ 　　　　｜CH$_3$　　　　　　｜CH$_3$	II. Ref. 5	1/10
R—CH : CH—C : CH—CH : C—C : CH—CH$_3$ 　　　　｜CH$_3$　　　　｜CH$_3$ ｜CH$_3$	III. Ref. 5	0
R—CH : CH—C : CH—CH : CH—C : CH—CH$_2$CH$_2$ 　　　　｜CH$_3$　　　　　　｜CH$_3$	IV. Ref. 5	0
R—CH : CH—C : CH—CH : CH—C : CH$_2$ 　　　　｜CH$_3$　　　　　　｜CH$_3$	V. Ref. 5	0
R—CH : CH—C : CH—CH : CH—CH : CH—CH$_2$OH 　　　　｜CH$_3$	VI. Ref. 6	1/30
R—CH : CH—C : CH—CH : CH—CH : C—CH$_2$OH 　　　　｜CH$_3$　　　　　　　　　　｜CH$_3$	VII. Ref. 6	0

Where R =
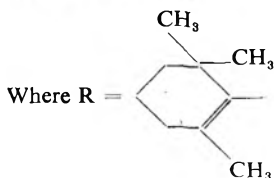
The findings of Morton *et al.*[7] in connection with retinenes gave support to VIII as the correct structure for vitamin A$_2$. Nothing has yet been published on compounds having the full vitamin A side chain attached to a different nucleus. Sobotka and Chanley[8] synthesised a cyclohexenyl analogue with two triple bonds in the side chain (IX); unfortunately, its biological activity was not published. More recently, Heilbron *et al.*[9] reported an acyclohexenyl analogue to vitamin A acid having a single

triple bond in the side chain (X) which was stated to have an activity 1/1000 that of Vitamin A. Since the presence of a triple bond next to the ring would have substantially changed the shape of the molecule, then, with the findings of Table I in mind, it may be asked whether this small activity was not due to a reduction *in vivo* into XI?

$$\text{—CH : CH—C : CH—CH : CH—C : CH—CH}_2\text{OH}$$
$$\qquad\qquad\ \ \underset{\text{CH}_3}{|}\qquad\qquad\quad\ \underset{\text{CH}_3}{|}$$

VIII.

$$\text{—C}{\equiv}\text{C—C : CH—C}{\equiv}\text{C—C : CH—CH}_2\text{OCH}_3$$
$$\qquad\quad\ \underset{\text{CH}_3}{|}\qquad\qquad\ \underset{\text{CH}_3}{|}$$

IX.

$$\text{—C}{\equiv}\text{C—C : CH—CH : CH—C : CH—COOH}$$
$$\qquad\quad\ \underset{\text{CH}_3}{|}\qquad\qquad\quad\ \underset{\text{CH}_3}{|}$$

X.

$$\text{—CH : CH—C : CH—CH : CH—C : CH—COOH}$$
$$\qquad\qquad\ \underset{\text{CH}_3}{|}\qquad\qquad\quad\ \underset{\text{CH}_3}{|}$$

XI.
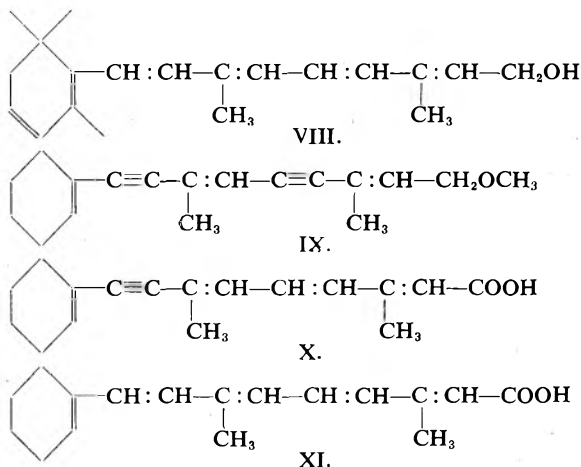
Attempts have been made in this laboratory to synthesise both the cyclohexenyl and the benzene analogue of vitamin A (XII, XIII). Owing to difficulties in preparing the key intermediate 1-Δ¹-cyclohexenyl-but-1-en-3-one, XII has not yet been obtained. However, the synthesis of the benzene analogue (XIII) has been achieved according to the route used by Isler *et al.*[2]

$$\text{—CH : CH—C : CH—CH : CH—C : CH—CH}_2\text{OH}$$
$$\qquad\qquad\ \underset{\text{CH}_3}{|}\qquad\qquad\quad\ \underset{\text{CH}_3}{|}$$

XII.

$$\text{—CH : CH—C : CH—CH : CH—C : CH—CH}_2\text{OH}$$
$$\qquad\qquad\ \underset{\text{CH}_3}{|}\qquad\qquad\quad\ \underset{\text{CH}_3}{|}$$
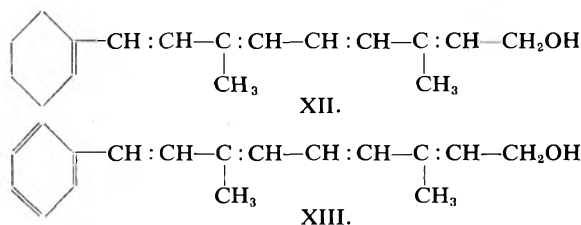
XIII.

This synthesis is summarised as follows, benzalacetone being used as the starting material.

$$\underset{\text{XIV.}}{\text{C}_6\text{H}_5\text{—CH : CH—}\underset{\underset{\text{CH}_3}{|}}{\text{C}}\text{ : O}}\ \xrightarrow[\substack{\text{Reformatzki reaction,}\\ \text{dehydration and saponification}}]{\text{BrCH}_2\text{CH : CH-COOEt}}\ \underset{\text{XV.}}{\text{C}_6\text{H}_5\text{—CH:CH—}\underset{\underset{\text{CH}_3}{|}}{\text{C}}\text{:CH—CH:CH—COOH}}$$

ClCH₂COOEt
Glycid ester condensation

$$\underset{\text{XVI.}}{\text{C}_6\text{H}_5\text{—CH : CH—}\overset{\overset{\displaystyle O}{\triangle}}{\text{C}}\text{—}\underset{\underset{\text{CH}_3}{|}}{\text{CHCOOEt}}}\ \xrightarrow{\text{saponification}}\ \underset{\text{XVII.}}{\text{C}_6\text{H}_5\text{—CH : CH—}\overset{\overset{\displaystyle O}{\triangle}}{\text{C}}\text{—}\underset{\underset{\text{CH}_3}{|}}{\text{CHCOOH}}}\ \xrightarrow{-\text{CO}_2}$$

$$\left[C_6H_5{-}CH:CH{-}\underset{\underset{CH_3}{|}}{CH}{-}CHO \right] \xrightarrow{\text{isomerisation}} C_6H_5{-}CH_2CH:\underset{\underset{CH_3}{|}}{C}{-}CHO$$

XVIII.   XIX.

$$HC\equiv C{-}\underset{\underset{CH_3}{|}}{C}:CH{-}CH_2OH \xrightarrow{\text{EtMgBr}} BrMgC\equiv C{-}\underset{\underset{CH_3}{|}}{C}:CH{-}CH_2OMgBr$$

XX.   XXI.

$$C_6H_5{-}CH_2CH:\underset{\underset{CH_3}{|}}{C}{-}\underset{\overset{OH}{|}}{CH}{-}C\equiv C{-}\underset{\underset{CH_3}{|}}{C}:CH{-}CH_2OH$$

XXII.

$\xrightarrow{\text{H}_2,\text{Pd/BaSO}_4}$

$$C_6H_5{-}CH_2CH:\underset{\underset{CH_3}{|}}{C}{-}\underset{\overset{OH}{|}}{CH}{-}CH:CH{-}\underset{\underset{CH_3}{|}}{C}:CH{-}CH_2OH$$

XXIII.

$\xrightarrow{\text{CH}_3\text{COCl, pyridine}}$

$$C_6H_5{-}CH_2CH:\underset{\underset{CH_3}{|}}{C}{-}\underset{\overset{OH}{|}}{CH}{-}CH:CH{-}\underset{\underset{CH_3}{|}}{C}:CH{-}CH_2OAc$$

XXIV.

$\xrightarrow[\text{(isomerisation, dehydration)}]{\text{refluxing with iodine}}$

$$C_6H_5{-}CH:CH{-}\underset{\underset{CH_3}{|}}{C}:CH{-}CH:CH{-}\underset{\underset{CH_3}{|}}{C}:CH{-}CH_2OAc$$

XXV.

$\xrightarrow{\text{saponification}}$

$$C_6H_5{-}CH:CH{-}\underset{\underset{CH_3}{|}}{C}:CH{-}CH:CH{-}\underset{\underset{CH_3}{|}}{C}:CH{-}CH_2OH$$

XXVI.

The Reformatzki reaction between benzalacetone and γ-bromocrotonic ester gave a hydroxy ester which was partially dehydrated upon distillation in high vacuum (10^{-4} mm. Hg pressure). After complete dehydration with anhydrous oxalic acid according to Arens and van Dorp[1], the product showed only a relatively low intensity of absorption in the ultraviolet region (max. 340mμ $E_{1 cm.}^{1 per cent.}$ 225) (Fig. 1, curve I). Saponification of the ester and recrystallisation of the crude gummy acid obtained from ether or acetone gave a small yield of a lemon yellow coloured crystalline acid. This acid, melting at 190° to 194°C., gave the correct analysis for carbon, hydrogen and active hydrogen required for 5-methyl-7-phenyl-hepta-2:4:6-trienoic acid. Light absorption (Fig.1, curve II),

in ethyl alcohol, showed a maximum at 342μ, log.ε 4·548, with a subsidiary peak at 249mμ. These figures are in close agreement with those expected. However, as the yield of this crystalline acid was small and subsequent work carried out with the crude acid was fruitless, a different approach to the problem was then undertaken.

FIG. 1.
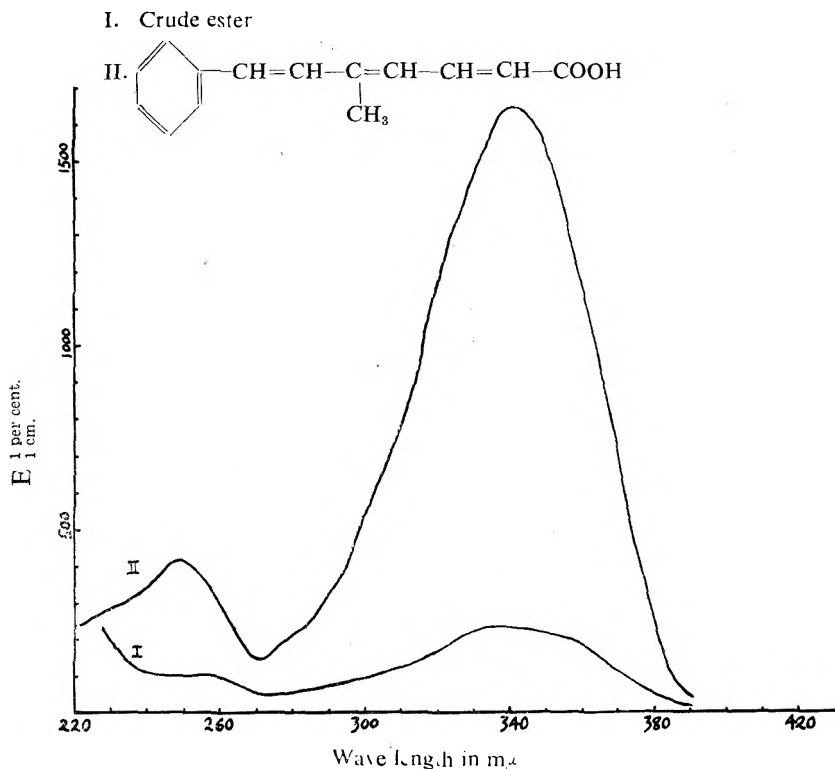
The glycid ester condensation was first carried out at − 7°C. by Darzen's method[10]. Fractionation of the condensation product gave a 23 per cent. yield of the glycid ester (XVI) as a viscous colourless liquid boiling at 130° to 134°C. at 0·5mm. Hg pressure. Analysis gave figures in close agreement with those required. The glycid ester was found to polymerise easily on heating, redistillation at the same temperature range and under the same pressure giving only 30 per cent. recovery, the rest forming a thick oily residue in the flask, non-distillable without decomposition and solidifying into a glassy mass on cooling.

The glycid ester thus obtained was saponified by cold alcoholic potassium hydroxide, formation of some potassium carbonate precipitate indicating a partial decarboxylation during this treatment. On acidification the free glycid acid (XVII) separated as reddish precipitate and was subjected to decarboxylation without further purification. Both Heilbron et al.[11] and Milas et al.[12] in their preparation of the C_{14} aldehyde, isolated

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the crystalline acid and decarboxylated by heating with copper or glass powder. However, with this benzene analogue, decarboxylation was found to be complete in about 15 minutes by simply heating the crude glycid acid over boiling water bath. Purification by fractionation gave about a 15 per cent. yield of the aldehyde based on the ester.

It has. been observed previously that the glycid ester (XVI) was very sensitive to heat. Polymerisation during saponification and decarboxylation may be responsible for the low yield of the aldehyde. By adopting Isler's modified method[4], the glycid ester was saponified *in situ* with alcoholic potassium hydroxide at low temperature. After working up in usual manner, a crop of pure aldehyde was obtained in an overall yield of 40 per cent. of the theoretical yield based on benzalacetone. This aldehyde, distilling at 70°C. under 0·05mm. Hg pressure showed the following characteristics: n $_D^{20°C.}$ 1·5537, d $_4^{20°C.}$ 1·0105, exaltation of molecular refraction 1·83 units. It gave analytical figures in close agreement with theory. The 2:4-dinitrophenylhydrazone occurred in red needles from chloroform, melting at 188° to 190°C. gave the correct analysis for nitrogen. The semicarbazone in leaflets from alcohol, had a melting-point 178° to 179°C. The thiosemicarbazone, needles from alcohol, had a melting-point, 132° to 132·5°C. According to classical concepts therefore, this aldehyde would be assigned formua XVIII.
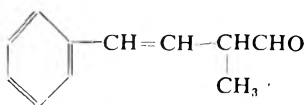
XVIII.
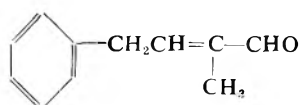
XIX.
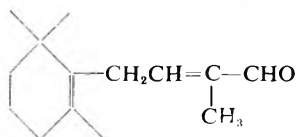
XXVI

But on spectroscopic examination (in ethyl alcohol), this aldehyde showed an absorption maximum at 229mμ, log.ε 4·214, indicating a substituted α:β-unsaturated aldehyde structure (XIX as 2-methyl-4-phenyl-crotonaldehyde. A small but definite elevation at 283 to 284 mμ, log. ε 3·375, may correspond to the so-called R band. Its semicarbazone showed an absorption maximum at 266 mμ, log. ε 4·536, thiosemicarbazone at 299 mμ, log.ε 4·409, both typical of the corresponding derivatives of α:β-unsaturated aldehydes in general[13]. This is in agreement with Heilbron's formula for the C_{14} aldehyde (XXVI)[6,11] which was opposed by Milas *et al.*[12]. The light absorption data for these two aldehydes and their derivatives together with those of citral are compared in Table II.

Further, if the aldehyde had the structure XVIII, it should show a maximum for the skeleton $C_6H_5 - C = C -$ at around 290 mμ[14]. Sayrene itself shows a maximum at 245 mμ, with two submaxima at 282 mμ and 290·4mμ[15]. The wide differences between these figures and those obtained suggest that the aldehyde in question does not contain the

TABLE II

						λmax. mμ	log
1. 2-Methyl-4-phenyl-crotonaldehyde (XIX) (Fig. 2)					...	229	4·214
2. C_{14} aldehyde (XXVI)[9]	...	...	...	...	...	230	4·25
3. Citral (commercial)[9]	...	...	...	...	...	232	4·05
4. Semicarbazone of 1 (Fig. 2)	...	...	...	...	...	266	4·536
5. Semicarbazone of 2[4]	...	...	...	...	...	265	4·47
						269	4·462
6. Semicarbazone of 3[9]	...	...	...	...	...	272	4·498
7. Thiosemicarbazone of 1 (Fig. 2)	...	...	...	...	299	4·409
8. Thiosemicarbazone of 2[9]	...	...	...	...	299	4·591
9. Thiosemicarbazone of 3[9]	...	...	...	...	303	4·66

$C_6H_5 - C = C -$ system in its structure. This affords a more definite answer pertaining to its structure and lends indirect support to Heilbron's formula for the C_{14} aldehyde.
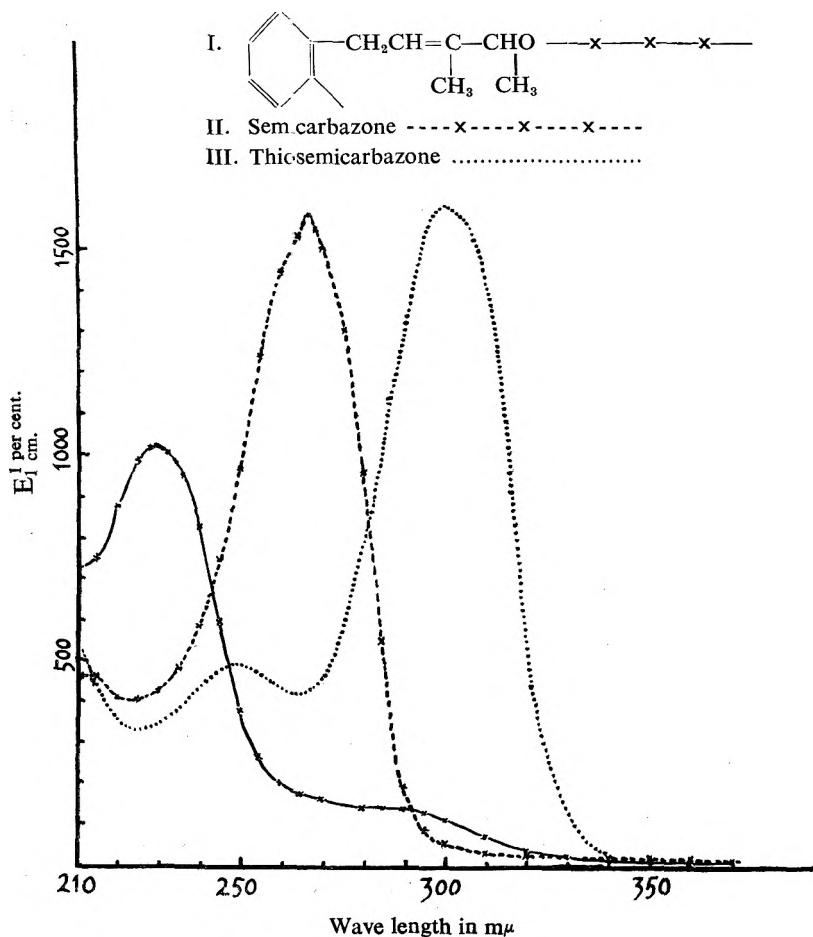
FIG. 2.

3-Methyl-pent-2-en-4-yn-1-ol (XX) was prepared per 3-methylpent-4-en-1-yn-3-ol by condensing methylvinyl ketone with sodium acetylide in liquid ammonia according to the method of Heilbron and Jones[16]. 3-Methylpent-4-en-1-yn-3-ol was found to have a refractive index $n_D^{17.5°C.}$ 1·4438 which is nearer to the figure given by Hennion and Leib[17] (n $^{20°C.}$ 1·4444) than that by Heilbron and Jones ($n_D^{15°C.}$ 1·4490) although the rearranged carbinol 3-methylpent-2-en-4-yn-1-ol had $n_D^{17°C.}$ 1·4850, the same as that given by Heilbron and Jones.

2-Methyl-4-phenylcrotonaldehyde was coupled with the Grignard compound of 3-methylpent-2-en-4-yn-1-ol (XXI) according to Isler et al.[4] In view of the fact that the reaction mixture was heterogeneous the mixture was refluxed with constant stirring for 7 hours to ensure complete reaction. After working up in usual way, the unchanged carbinol and aldehyde were removed in high vacuum. The residue so obtained was purified by partition between 75 per cent. aqueous methyl alcohol and light petroleum (40° to 60°C.) during which process the possible hydrocarbon formed was removed in the petroleum layer. The aqueous methyl alcoholic liquor was diluted with water and the oil separated was extracted with ether. Removal of the solvent gave the diol (XXII) as a viscous brownish oil in 78 per cent. yield. It showed the following characteristics: $n_D^{20°C.}$ 1·5756, $d_4^{19°C.}$ 1·0673, exaltation of molecular refraction 2·21 units. It gave analytical figures for carbon, hydrogen and active hydrogen in close agreement with theory. With antimony trichloride in chloroform it gave only a brownish black colouration. Its absorption spectrum showed inflections at 215 mμ and 230mμ which were probably due to the two isolated chromophores, the yn-en system and the benzene ring respectively. (Fig. 3, curve I.)

Semihydrogenation of the triple bond was carried out in the following way. A supported catalyst of palladium on barium sulphate (5 per cent. Pd) was prepared according to Organic Syntheses[18] and was partially inactivated by the use of Rosenmund-Zetzsche sulphur-quinoline poison[19,20]. The diol (XXII) was dissolved in 10 times its volume of methyl alcohol and the hydrogenation was carried out at atmospheric pressure. This hydrogenation process was found to be extremely slow in comparison with a control experiment on 3-methylpent-2-en-4-yn-1-ol, the catalyst used being very soon completely inactivated by impurities present in the diol. Three subsequent additions of fresh partially poisoned catalyst were made and the hydrogenation stopped after nearly 13 hours when the hydrogen uptake was 0·99 mol. per mol. of the diol. The product thus obtained after the removal of catalyst and solvent gave a slightly higher carbon and lower active hydrogen figures than those required for the diol (XXIII), indicating a partial dehydration during the prolonged shaking with the catalyst. This was further confirmed by the fact that with antimony trichloride in chloroform it gave a blue colour changing rapidly into violet then red. The spectroscopic results also showed a significant change. (Fig. 3, curve II.) The maxima at 215 mμ and 240 mμ apparently due to the diol (XXIII) itself, and corresponding to the diene conjugation and the benzene ring respectively,

F

Wave Length in mμ
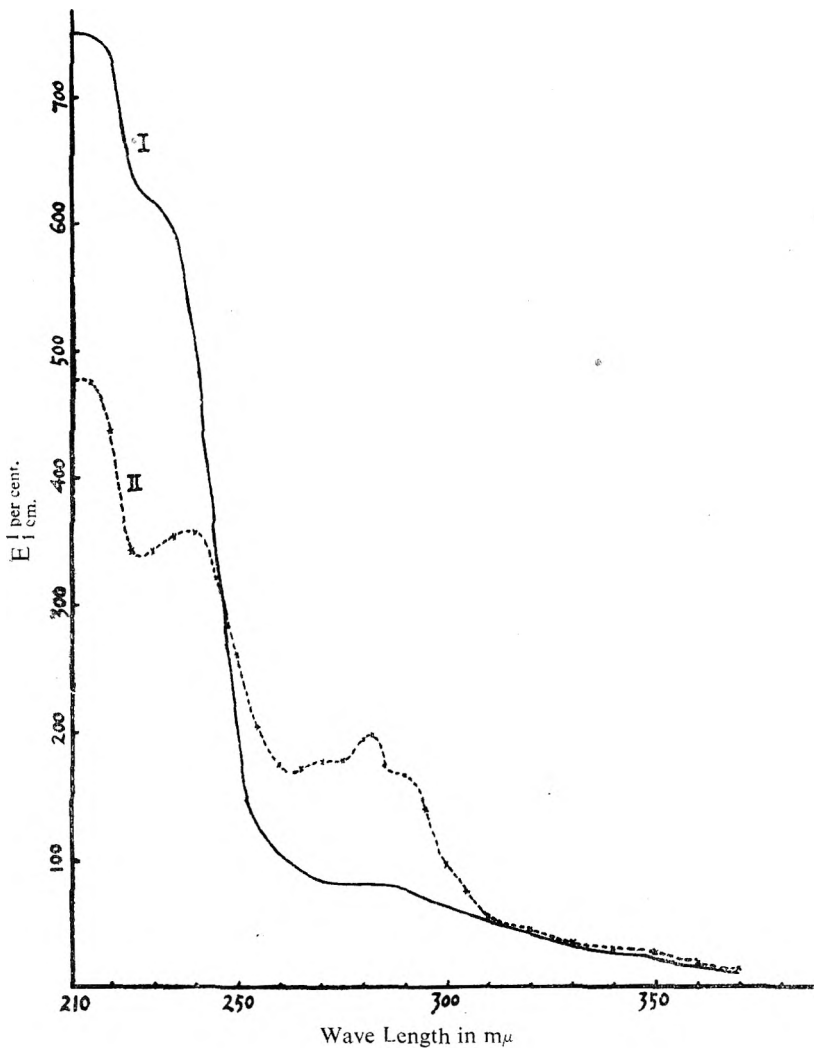
Fig. 3.

were lower than the corresponding inflections in curve I, whilst the newly formed peak at 282 mμ indicated the formation of a compound with 4 conjugated double bonds. This may possibly be explained by the formation of a hydrocarbon (XXVII) from the diol according to the following reaction:

$$\overset{\displaystyle OH}{C_6H_5-CH_2CH:C-\underset{|}{CH}-CH:CH-C:CH-CH_2OH} \qquad XXIII.$$

$$\downarrow$$

$$C_6H_5-CH_2CH:C-\underset{|}{\underset{CH_3}{CH}}:CH-CH:C-CH:CH_2 \qquad XXVII.$$

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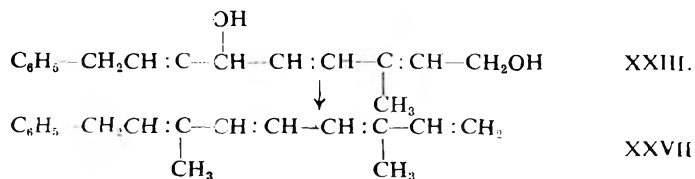
Partial acetylation of the diol (XXIII) was carried out by treating the diol with acetyl chloride in presence of dry pyridine. The crude acetylated product gave unsatisfactory analytical figures. With antimony trichloride in chloroform it gave a transient blue colour changing rapidly into violet and red as before. Spectroscopically, it was very similar to the diol before acetylation (Fig. 4, curve I), the slight proportional increase of intensity at 282 mμ probably indicating further dehydration along the suggested direction. This acetylated product was purified by solution in light petroleum (60° to 80°C.), the insoluble portion being a solid was probably a polymerised product. A light golden yellow coloured liquid was obtained from the petroleum fraction corresponding to 66 per cent. of the crude material, the analytical figures being in fair agreement with those required for the monoacetate of the diol (XXIV). This purified monoacetate was used for the following dehydration.

The dehydration was carried by the method used by Isler *et al.*[4] in the synthesis of vitamin A. A solution of the acetate in light petroleum (100° to 120°C.) after being stabilised with α-tocopherol, was refluxed with iodine for 1 hour under nitrogen. The product obtained was examined spectroscopically. (Fig. 4, curve II.) A new absorption peak produced at 325 mμ, with $E_{1\ cm.}^{1\ per\ cent.}$ 245, indicated the formation of 3:7-dimethyl-9-phenylnona-2:4:6:8-tetraene-1-ol acetate (XXV). However, active hydrogen determination gave a figure corresponding to the presence of about 36 per cent. of XXIV, which is thought to be responsible for part of the spectrum.

A number of ways are known to achieve this dehydration. In the synthesis of vitamin A ether, Milas *et al.*[17] described the use of *p*-toluenesulphonic acid, pyridine hydrobromide in presence of pyridine, alcoholic potassium hydroxide, phosphorus tribromide or thionyl chloride in conjunction with pyridine, or sodamide in liquid ammonia. The iodine method has the advantage that it catalyses *cis-trans* isomerisation[22,23], although it has been reported in the literature[24,25,26,27] that dehydration usually resulted in a *trans* bonding, semihydrogenation of the triple bond gave in most instances a *cis* double bond[28]. However, the broadness of the absorption band produced by the product suggests that stereoisomers were probably present.

Saponification of the acetate gave a product containing the free carbinol, 3:7-dimethyl-9-phenylnona-2:4:6:8-tetraene-1-ol (XIII). It showed an absorption maximum at 329 mμ with $E_{1\ cm.}^{1\ per\ cent.}$ 254 (Fig. 4, curve III). Spectroscopically, a benzene ring effects approximately the same bathochromic shift to the absorption peak of a polyene compound as does an extra conjugated double bond. This was found to be the case in $\alpha : \beta$-unsaturated ketones by Wilds *et al.*[29] Thus, with a ring double bond, methyl substituted, β-ionone has a maximum (at 293 mμ) 4 units towards the longer wave length than benzalacetone (at 289 mμ). 1-Vinylcyclohexene-1 on the other hand, has a maximum (at 230 mμ)[30] 10 units towards the shorter wave length than styrene (at 240 mμ)[31]. Hence, it was expected that the maximal absorption of the carbinol (XIII) should be in the neighbourhood of that of vitamin A.

I. ——x——x——x——
II. ----x----x----x--
III. ....

$E_{1\ cm.}^{1\ per\ cent.}$

Wave length in mμ
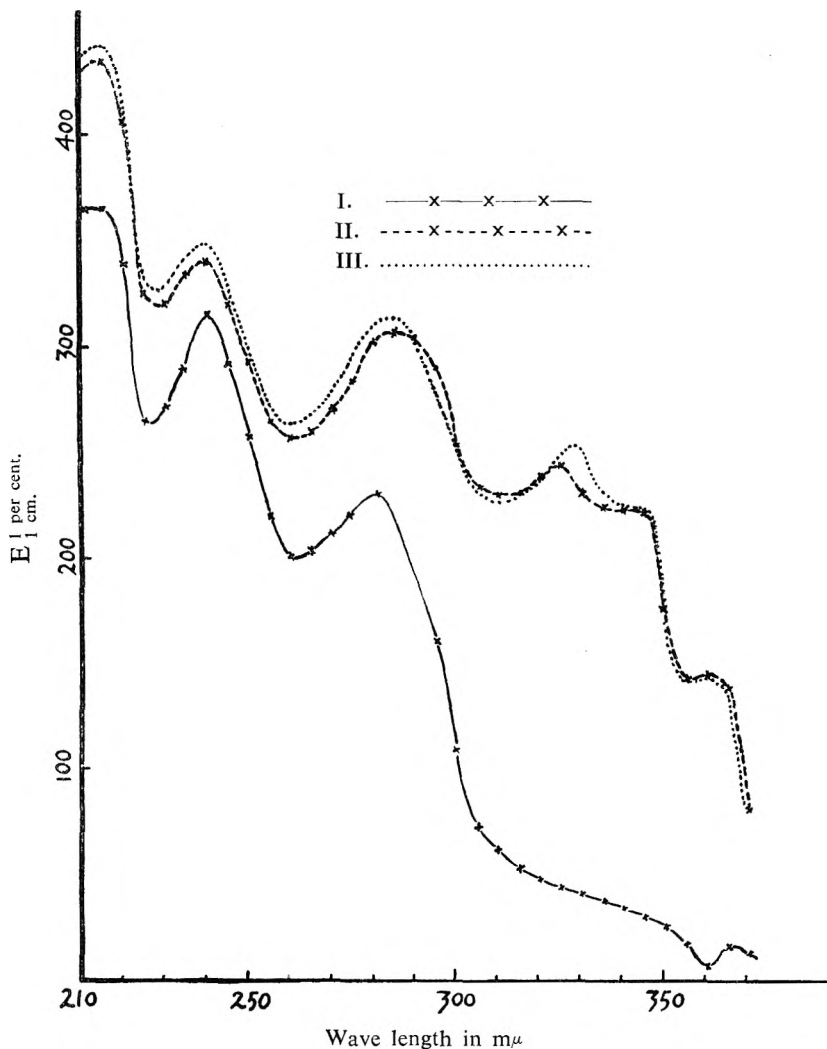
FIG. 4.

It is to be regretted that owing to the great instability of the product obtained, further purification of the sample by chromatographic means was not attainable. The bulk of the sample, stored under nitrogen in the dark, deteriorated during the course of its spectral and biological studies as shown by a loss of the specific absorption properties. The acetate, which was tested biologically, was found to be completely inactive.

Examination of the structure of XIII reveals its differences from vitamin A in two respects: (a) the presence of the benzene ring which brings the whole molecule into coplanarity and hence different from vitamin A slightly in its spatial arrangements. On the other hand, the

presence of a stable benzene nucleus in the molecule may modify its chemical activity. (b) the absence of the three methyl substituent groups from the ring. They may have a certain specific effect. Therefore it can be concluded that the polyene carbinol side chain alone in the vitamin A molecule is not sufficient, though necessary, for producing vitamin A activity. These results stimulate interest in the synethesis of the corresponding cyclohexenyl analogue of the vitamin (XII) in order to determine whether it possesses any biological activity.

<div align="center">EXPERIMENTAL</div>

All absorption spectroscopic measurements were carried out on solutions in ethyl alcohol. Melting-points are uncorrected.

Benzalacetone. Prepared according to Organic Syntheses[32], having m.pt. 42°C.

Ethyl γ-bromocrotonate. Prepared according to the method of Ziegler et al.[33]. The fraction having b.pt. 91° to 93°C./10mm. Hg. pressure was used.

Reformatzki condensation of benzalacetone and ethyl γ-bromocrotonate Benzalacetone (146 g.), ethyl γ-bromocrotonate (193 g.) and benzene (sodium dried, 1000 ml.) were mixed together with zinc wool (washed with acetone and dried, 65·4 g.) and a small crystal of iodine. The whole was heated under a reflux condenser with stirring over a steam bath to start the reaction which occurred a few minutes after the refluxing had started. The heating was then interrupted till the reaction had subsided, the refluxing being then continued for another 4 hours. After cooling the unreacted zinc was collected (12·8 g.). The benzene solution of the complex was decomposed with crushed ice and diluted acetic acid (5 per cent.). Benzene extraction gave 180 g. of a reddish oily liquid after removal of solvent. By subjecting this liquid to distillation at 0·01mm. Hg pressure, partial dehydration occurred at a bath temperature of 40°C. A fraction of benzalacetone, identified by its 2:4-dinitrophenylhydrazone, was recovered at bath temperature 106° to 110°C. (53·7 g.). The distillation was continued at 130°C. for a further half-hour when only a small amount of unidentified material distilled over. The non-distillable material was subjected to high vacuum distillation in a modified short path still of the cold finger type. The material distilled over at 115° to 130°C. (bath temperature) at 10^{-4}mm. Hg pressure and consisted of a mixture of the hydroxy ester and the dehydrated ester. Active hydrogen (Zer.) corresponding to 0·445 H.

Ethyl 5-methyl-7-phenylhepta-2:4:6-trienoate (crude). The partially dehydrated ester (50 g.) was mixed with half its weight of anhydrous oxalic acid and heated at 110°C. under reduced pressure (10mm. Hg) for 1½hours. The product was extracted with benzene, the benzene extract being washed with solution of sodium bicarbonate and water. After the removal of benzene, the product was distilled in high vacuum, when the dehydrated ester distilled over at 93° to 98°C. 10^{-4} to 10^{-5}mm. Hg pressure. Weight: 38 g. Active hydrogen (Zer.): negligible. Light absorption:

(Fig. 1, curve I) maximum 340mμ, $E_{1\,cm.}^{1\,per\,cent.}$ 225; inflection 250 mμ. $E_{1\,cm.}^{1\,per\,cent.}$ 105. Refractive index: $n_D^{20°C.}$ *ca.* 1·55.

5-Methyl-7-phenylhepta-2 : 4 : 6-trienoic acid. The dehydrated ester (21 g.) was saponified by shaking with alcoholic potassium hydroxide (7 g. of potassium hydroxide in 77 ml. of alcohol (90 per cent.)) overnight. The soap solution was diluted with water (500 ml.) and extracted with ether which yielded on evaporation only about 0·5 g. of a neutral fraction. The soap solution was then acidified with dilute acetic acid (5 per cent.). The crude acid separated as a gummy precipitate which solidified on standing. By dissolving the crude acid in ether and cooling in a refrigerator overnight, a lemon yellow coloured crystalline acid melting at 190° to 194°C. was obtained. Its colour darkened on standing in air, and it was therefore kept in an atmosphere of nitrogen. Yield: 0·8 g. from about 15 g. of crude acid. Found: C. 77·1; H, 6·56; $C_{14}H_{14}O_2$ requires C, 78·5; H. 6·54 per cent. Active hydrogen (Zer.): 1·05 H. Light absorption (Fig. 1, curve II) Maxima: 342 mμ, $E_{1\,cm.}^{1\,per\,cent.}$ 1650, log.ε 4·548; 249 mμ, $E_{1\,cm.}^{1\,per\,cent.}$ 418, log.ε 3·952. The bulk of the acid was non-crystallisable.

Glycid ester condensation of benzalacetone and ethyl chloroacetate. The general method given by Darzens was followed[10]. Benzalacetone (28 g.) and ethyl chloroacetate (freshly distilled, 23·8 g.) were mixed together in a flask and the mixture cooled to -7°C. Alcohol free sodium ethoxide (13 g.) was added in small portions with constant stirring over a period of about $1\frac{1}{2}$ hours. The mixture was stirred at room temperature overnight, then heated over a water bath for 1 hour. After cooling, 100 g. of crushed ice was added followed by the gradual addition of dilute acetic acid (60 ml. of 11 per cent. acid). Extraction with ether and subsequent removal of the solvent gave 43 g. of material which was fractionated. The glycid ester distilled over at 133° to 137°C./0·5 mm. Hg. pressure as a colourless viscous oil. Yield: 10 g. (23 per cent. of theory). It had refractive index: $n_D^{33°C.}$ 1·5401. Found: C, 72·41; H, 6·74; $C_{14}H_{16}O_3$ requires C, 72·41; H, 6·95 per cent. Saponification value gave its molecular weight as 227: $C_{14}H_{16}O_3$ requires 232.

2-Methyl-4-phenylcrotonaldehyde (XIX). The glycid ester (26·3 g.) in alcohol (40 ml.) was saponified with alcoholic potassium hydroxide (142 ml. of 1·8 N.) by shaking in an atmosphere of nitrogen for 2 hours and then leaving to stand overnight. A small amount of potassium carbonate precipitate formed during this treatment. After dilution with water and acidification, the free glycid acid precipitated as a red coloured gummy solid. This crude acid was not separated and further purified, but the whole mixture was heated over a boiling water-bath. Decarboxylation occurred smoothly and was completed in about 15 minutes when the solid acid changed into an oily liquid. After extraction with ether, the ethereal extract was washed with water and fractionated yielding mainly the crude 2-methyl-4-phenylcrotonaldehyde at 102° to 110°C./0·3 mm. Hg pressure as a pale yellow liquid. Yield, 3 g. (16 per cent. of theory based upon the glycid ester).

2-Methyl-4-phenylcrotonaldehyde (*pure*) *by improved method.* The method used by Isler *et al.*[4] was followed. Benzalacetone (56 g.) and ethyl chloroacetate (46·5 g.) were mixed in a flask cooled in a solid carbon-dioxide-acetone bath. Sodium ethoxide (26 g.) was added in small portions with stirring as before, the whole being left overnight at room temperature. Alcoholic potassium hydroxide (220 ml. of 1·8 N.) was added to the mixture gradually with constant stirring, cooling as before. After being stirred for 3 hours, the whole was left over the week-end. Water (600 ml.) was then added with cooling followed by extraction with ether. The aqueous saponaceous liquor on acidification gave only a negligible amount of oily liquid, which indicated almost complete decarboxylation of the glycidate under these conditions. The ethereal extract, after being washed with water and dried, was distilled to remove the solvent, giving 53·3 g. of product which was fractionated:

Fraction 1: 80° to 82°C./0·05 mm. Hg pressure, 25 g. $n^{22°C.}$ 1·5539.

Fraction 2: 84° to 92°C./0·05 mm. Hg pressure, 3 g. $n^{22°C.}$ 1·5549.

Fraction 3: 110° to 40°C./0·05 mm. Hg pressure, 3 g. $n^{22°C.}$ 1·5660.

Fraction 1 was apparently the main crop of the required aldehyde which on redistillation at 70°C./0·05 mm. Hg gave a yield of 22 g. of the pure aldehyde (about 40 per cent. of theory overall) The aldehyde gave the following constants: $n_D^{20°C.}$, 1·5537; $d_4^{20°C.}$, 1·0105; molecular refraction: found 50·77, calculated for C_{11}, H_{12}, O'', F_4 48·94, exaltation 1·83 units. Found: C, 82·11; H, 7·69; $C_{11}H_{12}O$ requires C, 82·50; H, 7·50 per cent. Semicarbazone, leaflets from alcohol, m.pt. 178° to 179°C.; thiosemicarbazone, needles from alcohol, m.pt. 132° to 132·5°C.; 2:4-dinitrophenylhydrazone, red needles from chloroform, m.pt. 188° to 190°C. Found: N, 16·1; $C_{17}H_{16}N_4O_4$ requires N, 16·47 per cent.

Light absorption data: (in ethyl alcohol) (Fig. 2).

The aldehyde: Maxima: 229 mμ, $E_{1 cm.}^{1 per cent.}$ 1021, log ε 4·214; 283 to 284 mμ, $E_{1 cm.}^{1 per cent.}$ 142, log ε 3·357.

The semicarbazone: Maximum 266 mμ, $E_{1 cm.}^{1 per cent.}$ 1581, log ε 4·536.

The thiosemicarbazone: Maxima 299 mμ, $E_{1 cm.}^{1 per cent.}$ 1601, log ε 4·409; 249 mμ, $E_{1 cm.}^{1 per cent.}$ 490, log ε 3·895.

Fraction 2 on redistillation yielded mainly the same aldehyde distilling at 70° to 72°C./0·05 mm. Hg pressure.

Fraction 3 yielded a 2:4-dinitrophenylhydrazone, but was apparently a complex mixture and was not investigated further.

3-Methylpent-4-en-1-yn-3-ol was prepared by the method given by Heilbron and Jones[14]. The product on careful fractionation through a Widmer column gave the pure carbinol distilling at 66° to 66·5°C./50 mm. Hg pressure. It had refractive index: $n_D^{17·5°C}$ 1·4438 (Lit., $n_D^{15°C}$, 1·4490[14]; $n^{20°C.}$, 1·4444[15]). Active hydrogen: (Zer.) 1·09 H at room temperature, 2·03 H after heating at 100°C.

3-Methylpent-2-en-4-yn-1-ol (XX) was obtained by anionotropic rearrangement of 3-methylpent-4-en-1-yn-3-ol according to Heilbron and

Jones[14]. The carbinol distilled at 77° to 78°C./18 mm. Hg pressure. Refractive index $n_D^{17°C.}$ 1·4850 (Lit., $n_D^{16°C.}$ 1·4850[14]). Active hydrogen (Zer.); 1·93 H. after warming. α-Naphthylurethane: m.pt. 118° to 119°C. (Lit., 119°C.[16]).

3:7-Dimethyl-9-phenylnona-2:7-dien-4-yn-1:6-diol (XXII).   The condensation was carried out in a similar way to that used by Isler *et al.*[1] To the Grignard compound of 3-methylpent-2-en-4-yn-1-ol (XXI) (from 5·1 g. of the carbinol) in ether was added a solution of 2-methyl-4-phenyl-crotonaldehyde (8·0 g.) in ether (12·5 ml.) over a period of 15 minutes, the whole being cooled in an ice water bath.   Vigorous stirring was maintained and a slow stream of nitrogen was passed into the apparatus all the time.   After the completion of the addition of the aldehyde, the whole mixture turned into a stiff mass insoluble in ether and was then refluxed over a warm water-bath with efficient stirring when the mass gradually softened.   The refluxing was continued for 7 hours.   It was then decomposed by shaking with crushed ice and ammonium chloride solution in nitrogen overnight.   The product was then extracted with ether, the ethereal extract being dried and fractionated.   About 1 ml. of the 3-methylpent-2-en-4-yn-1-ol and a small amount of the 2-methyl-4-phenylcrotonaldehyde were recovered.   The product, after being deprived of low-boiling material under 0·04 mm. Hg pressure over a water-bath for 1 hour, was purified by a partition between light petroleum and aqueous methyl alcohol: The product was dissolved in methyl alcohol (100 ml. of 75 per cent.) and extracted with light petroleum (3 quantities, each of 30 ml.).   The petroleum fraction which contained some hydrocarbon was not investigated further.   The aqueous methyl alcohol fraction was diluted with water (400 ml.) and extracted with ether (4 quantities, each of 70 ml.).   After the removal of the last trace of solvent from the ethereal extract at 70° to 80°C./0·05 mm. Hg pressure, 3:7-dimethyl-9-phenylnona-2:7-dien-4-yn-1:6-diol was obtained as a viscous brownish liquid. Yield: 10 g. (78 per cent. of theory).   It had the following characteristics: $n_D^{20°C.}$ 1·5756; $d_4^{19°C.}$ 1·0673: Molecular refraction : found 79·43, calculated for $C_{17}H_{20}O_2'$ F_3 77·22; exaltation : 2·21 units.   Found C, 79·86; H. 7·95; $C_{17}H_{20}O_2$ requires C, 79·68; H, 7·88 per cent.   Active hydrogen (Zer.): 2·04 H.   Light absorption: (Fig. 3, curve I) inflections at 215 mμ, $E_{1\ cm.}^{1\ per\ cent.}$ about 750: 230 mμ $E_{1\ cm.}^{1\ per\ cent.}$ about 615.

Partially poisoned catalyst.   A supported catalyst of 5 per cent. palladium over barium sulphate (6 g., prepared according to Organic Syntheses[15]) suspended in purified alcohol (30 ml.) was treated with quinoline sulphur poison[16] (0·6 ml.).   After being stirred for ½ hour, the catalyst was collected on a filter, washed with a little alcohol and dried *in vacuo.*

3:7-Dimethyl-9-phenylnona-2:4:7-trien-1:6-diol (XXIII).   3:7-Di-methyl-9-phenylnona-2:7-dien-4-yn-1:6-diol (9 g.) was dissolved in methyl alcohol (90 ml.) to which the partially poisoned catalyst (1 g.) was

added. Hydrogenation was carried out under approximately atmospheric pressure. However, after the hydrogenation had proceeded slowly for about 4 hours, the rate of absorption of hydrogen became very slow. Fresh additions of catalyst were made and the hydrogenation stopped when 0.99 mol. proportion of hydrogen was absorbed. Total amount of catalyst used was 2·3 g. The catalyst was removed from the solution and the solvent distilled off in nitrogen, the final trace of volatile matter being removed at 80° to 90°C./0·05 mm. Hg pressure. Yield: 9·0 g. of brownish liquid. Found: C. 80·75; H, 8·84; $C_{17}H_{22}O_2$ requires C, 79·02; H, 8·58 per cent. It had the following characteristics: $n_D^{17°C.}$ 1·5673, $d_4^{17°C.}$ 1·039. Molecular refraction : found : 81·15, calculated for $C_{17}H_{22}O'_2F_6$: 78·75; exaltation 1, 2·40 units. Active hydrogen (Zer.) : found 0·63 per cent., $C_{17}H_{22}O_2$ requires 0·78 per cent. for 2H. With chloroformic solution of antimony trichloride it gave an instantaneous blue coloration changing rapidly through violet to red. Light absorption: (Fig. 3, curve II) Maxima 215 mμ, $E_{1\,cm.}^{1\,per\,cent.}$ 357; 282 mμ, $E_{1\,cm.}^{1\,per\,cent.}$ 197.

3 : 7-Dimethyl-9-phenylnona-2:4:7-trien-1 : 6-diol monoacetate (XXIV). The diol obtained above (6·5 g.) was dissolved in a mixture of benzene (40 ml.) and dry pyridine (40 ml.). The solution was cooled in a solid carbon dioxide-acetone bath, and freshly distilled acetyl chloride (2·3 g.) was added drop by drop with stirring in nitrogen. After the completion of the addition, the cooling bath was removed and the whole was stirred at room temperature for $\frac{3}{4}$ hour, then refluxed for 1 hour. The mixture was then cooled to below 0"C., decomposed with water and extracted with benzene. Removal of solvent gave the crude monoacetate as a brownish viscous liquid. Yield: 7·4 g. Light absorption: (Fig. 4, curve I) Maxima 215 mμ $E_{1\,cm.}^{1\,per\,cent.}$ 367; 240 mμ, $E_{1\,cm.}^{1\,per\,cent.}$ 316; 282mμ, $E_{1\,cm.}^{1\,per\,cent.}$ 232.

Purification of the monoacetate. The crude acetate (6·6 g.) was extracted repeatedly with hot petroleum (100°C.), the residue insoluble in petroleum being a dark browish gummy solid, probably a polymerised product. The petroleum extract was evaporated to remove the solvent and a light golden yellow liquid was obtained. Found: C. 75·06; H, 7·63; $C_{19}H_{24}O_3$ requires C, 75·97; H, 8·05 per cent. Refractive index: $n_D^{20°C.}$ 1·557. Active hydrogen (Zer.): 1·1 H.

3 : 7-Dimethyl-9-phenylnona-2 : 4 : 6 : 8-tetrane-1-ol   acetate (XXV). The dehydration was carried out according to Isler et al.[4] The monoacetate obtained from above (4·2 g.), α-tocopherol (40 mg.), were dissolved in light petroleum (80° to 100°C., 70 ml.). Iodine (40 mg.) in light petroleum (10 ml.) was added and the whole was refluxed for 1 hour. After cooling, the solution was washed with solution of sodium thiosulphate and then with water. Removal of solvent gave a dark brown oil. Yield: 3·8 g. With antimony trichloride in chloroform a transient blue colour which changed rapidly through violet to red was produced. Light absorption (Fig. 4, curve II) Maxima 215 mμ, $E_{1\,cm}^{1\,per\,cent.}$ 435; 240 mμ, $E_{1\,cm.}^{1\,per\,cent.}$ 340; 282 mμ, $E_{1\,cm.}^{1\,per\,cent.}$ 306; 325 mμ, $E_{1\,cm.}^{1\,per\,cent.}$ 245. Active hydrogen: 0·36 H.

3 : 7-*Dimethyl*-9-*phenylnona*-2 : 4 : 6 : 8-*tetraene*-1-*ol* (XIII). The acetate (0·5 g.) was dissolved in absolute alcohol (3 ml.), to which, with stirring and cooling, was added alcoholic potassium hydroxide (3 ml. of N) in an atmosphere of nitrogen. After standing overnight, the solution was diluted with water (50 ml.) and extracted with ether. The ethereal extract was washed and dried, and the solvent removed. A brownish viscous oil was obtained, weighing 0·35 g. With antimony trichloride in chloroform : An instantaneous blue coloration which changed rapidly through violet to red. Light absorption : Fig. 4, curve III) Maxima 215 mμ, $E_{1\,cm.}^{1\,per\,cent.}$ 446; 240 mμ, $E_{1\,cm.}^{1\,per\,cent.}$ 350; 282 mμ, $E_{1\,cm.}^{1\,per\,cent.}$ 307; 329 mμ $E_{1\,cm.}^{1\,per\,cent.}$ 254.

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ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

Benadryl and Pyribenzamine Hydrochlorides, Identification and Differentiation of. T. J. Haley. (*J. Amer. pharm. Ass., Sci. Ed.*, 1948, **37**, 294.) The reactions of 12 precipitants and 16 colorimetric reagents with benadryl hydrochloride (diphenhydramine hydrochloride) and with pyribenzamine hydrochloride (tripelennamine hydrochloride) are described. Only chloroplatinic acid, 5 per cent., could be used by precipitation for identigfication and differentiation between these two. Benadryl gives a granular orange precipitate of leaf-like crystals in crosses with some cigar-shaped crystals. Pyribenzamine gives a granular orange precipitate of rosettes and sheaves of flat plates on drying. The colour reactions are distinctive; with concentrated sulphuric acid, benadryl gives an orange colour and pyribenzamine a greenish yellow colour; complete destruction of the organic compounds by the strong acid results in a dark brown to black solution unsuitable for qualitative analysis. With potassium dichromate and concentrated sulphuric acid, benadryl forms a yellow solution and pyribenzamine a brown solution. Resorcinol and concentrated sulphuric acid gives an orange and then reddish orange colour with benadryl, which becomes wine-coloured when diluted with water; the same reagent gives a yellowish green and then deep green colour with pyribenzamine which becomes olive-green on dilution with water. Furfurol 1 per cent. overlay sulphuric acid gives an orange-brown colour changing to yellow-green on shaking with benadryl, and a black colour which does not alter on shaking with pyribenzamine. Mandelin's reagent gives a red colour with oily red globules with benadryl, and a chocolate-brown colour with pyribenzamine; Marquis' reagent, yields a colour change with benadryl from canary-yellow to reddish-orange to chocolate-brown, and from red to deep reddish-brown with pyribenzamine; Mecke's reagent, gives a canary-yellow then reddish-yellow colour with benadryl, and a nut-brown colour with pyribenzamine; and Fröhde's reagent gives a canary-yellow colour followed by orange and then red with benadryl, but with pyribenzamine yields a pale pink followed by a deep rust colour.

L. H. P.

Progesterone, Photometric Determination of. E. Diding. (*Svensk. Farm. Tidskr.*, 1949, **53**, 269.) The method is based on the formation of a red dinitrophenylhydrazone, soluble in chloroform. The colour is stable for at least 24 hours. Details are as follows: 5 ml. of an alcoholic solution, containing 0·25 to 1·25 mg. of progesterone is treated with 3·0 ml. of a freshly-prepared 0·25 per cent solution of 2·4-dinitrophenylhydrazine in 2M hydrochloric acid. The mixture, in a covered beaker, is heated on the water-bath for 15 minutes, then treated with 10 ml. of 2M hydrochloric acid, and heated for a further 30 minutes to remove the alcohol. The precipitate is transferred to a sintered glass filter, washed with hydrochloric acid, and then with water. After drying *in vacuo*, the dinitrophenylhydrazone is dissolved in chloroform to 100 ml., and the extinction is determined at 440 mμ. For solutions in oil, 5 ml. of the solution is dissolved in 10 ml. of hexane and extracted with 4 quantities, each of 5 ml., of alcohol

(90 per cent.). The alcoholic extracts are washed with 20 ml. of hexane, which is then washed with 2 quantities, each of 5 ml., of alcohol. The alcoholic solutions are filtered and evaporated to dryness, and the residue is dissolved in alcohol and treated as before. G. M.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

nor-Adrenaline in Adrenal Medulla, Evidence for Occurrence of. M. Goldenberg, M Faber, E. J. Alston and E. C. Chargaff. (*Science*, 1949, **109**, 534.) By paper chromatography using phenol saturated with water as eluent in an atmosphere of hydrogen chloride it has been found that samples of U.S.P. Reference Standard Epinephrine contained 12 to 18 per cent. of nor-adrenaline and that one sample contained as much as 36 per cent. The adrenaline fractions from three chromaffin tissue tumours were found to contain 50 to 90 per cent of nor-adrenaline. It is pointed out that adrenaline and nor-adrenaline differ significantly in their pharmacological actions both as regards effect on cardiac output and on carbohydrate metabolism. If it is assumed that natural adrenaline as secreted by the adrenal gland maintains a constant content of nor-adrenaline, present concepts of adrenal secretion remain valid. If, however, under varying physiological conditions the nor-adrenaline content of the secreted natural adrenaline varies then it is considered that current views of the physiology of the adrenal medulla may have to be modified. Under pathological conditions such as in pheochromacytoma, hæmodyamic effects and the influence on carbohydrate metabolism are profoundly altered by the high content of nor-adrenaline in the secreted medullary hormone. The biological assay of tumour extracts is discussed. F. H.

BIOCHEMICAL ANALYSIS

Barbiturates in Tissue; Determination by Ultraviolet Absorption Spectrophotometry. G V. R. Born. (*Biochem. J.*, 1949, **44**, 501.) A procedure based on ultraviolet spectrophotometry is described for the quantitative determination of very small amounts of barbiturates in tissues and blood. The tissue is homogenised and proteins precipitated by ethyl alcohol. The barbiturate in the acidified protein-free filtrate is extracted with ether and passed into alkali. To two samples of this alkaline solution are added phosphate or borate solutions which bring the pH to different known values. The extinctions at 23 mμ of the resulting solutions are measured and from these the concentration of barbiturate in the tissue can be determined by calculation. Complete elimination of contaminants is not attemped. The difficulty due to the simultaneous extraction of barbiturate and impurities absorbing in the same region of the ultraviolet is overcome by the use of differential spectrophotcmetry, depending on extinction-pH relationships. The procedure is rapid, accurate and sensitive, permitting the extinction of concentrations of barbiturates as low as 1 to 2 μg./ml. in pure solution.

S. L. W.

Nicotinamide in Biological Materials, Fluorimetric Estimation of. D. K. Chaudhuri and E. Kodicek. (*Biochem. J.*, 1949, **44**, 343.) The material under examination (5g.) is cut finely, ground with sand and 0·1N hydrochloric acid (1 to 2 ml.), transferred with 40 ml. of water to a 100-ml. beaker on a boiling water-bath and heated for 30 minutes. After

cooling, hydrochloric acid is added to pH 2, the mixture is centrifuged, the residue washed with 10 ml. of 0·1N hydrochloric acid and again centrifuged. The combined liquids are adjusted to a known volume (usually 40 ml.) with 0·1N hydrochloric acid, 6 ml. of a freshly prepared 25 per cent. solution of metaphosphoric acid is added and the liquid centrifuged after standing for 5 to 10 minutes. The clear solution is adjusted to pH 9·4 to 9·6, heated on a boiling water-bath for 30 minutes, cooled, adjusted to pH 7·2 and made up to 50 ml.; after filtration (Whatman No. 5) it is then treated with cyanogen bromide solution. Three determinations are made—a blank, the unknown filtrate (containing 5 to 25 µg. of nicotinamide), and the unknown to which is added an internal standard (25 µg. nicotinamide). After mixing, the three solutions are heated in a water-bath for 4 minutes at 56° to 58°C., cooled and made up to 15 ml. with water. 5N sodium hydroxide (8 ml.) is added, the volume made up to 30 ml. with water and the fluorescence read after 45 minutes at room temperature in the dark. Details of the calculation and of the reproducibility of the results are given; the method estimates the total nicotinamide content, including the free and bound forms. Specific and reproducible results were obtained for biological materials and for cereals, and the results agreed well with the reported micro-biological values. Practically all the vitamin in rat organs and muscles seemed to be present in the form of the amide, bound or free. In bran, no nicotinamide was detected before or after digestion. The breakdown product of the " precursor " of nicotinic acid present in bran appeared to be the free acid and not the amide. Yeast and wheat germ contained about 50 per cent. of the vitamin present in the amide form, bound or free.

R. E. S.

CHEMOTHERAPY

Analgesic Compounds, Potential, Preparation of. D. J. B r o w n, A. H. C o o k and I. M. H e i l b r o n. (*J. chem. Soc.,* 1949, Supp. 1, S.106, S 111 and S 113.) In a search for more potent analgesics than amidone analogues have been prepared and examined. Attempts to introduce thiazolyl groups into 1-diethylamino-3-phenylpentan-4-one were unsuccessful. 4-Methyl-2-(3′-diethylamino-1′-phenylpropyl) thiazole and related componds were prepared from the corresponding γ-dialkylamino-α-phenylthicbutyramide but it was not found possible to introduce ester or ketone groupings on the *tert*-carbon atom. 5-Carbethoxy-4-methyl-2-(3′-diethylamino-1′-phenylpropyl) thiazole and 4-methyl-2-(4′-diethylamino-2′-acetyl-2′-phenylbutyl)thiazole, obtained as viscous oils, were without significant analgesic action. Introduction of basic groups into 4-phenyl- and 4-methyl-2-α-carbethoxybenzyl thiazole failed to yield analgesic properties. Direct carbethoxylation or propionylation of 2-benzylthiophen followed by introduction of a basic side chain yielded products having analgesic properties. Thus 2-(3′-diethylamino-1′-carbethoxy-1′phenylpropyl)thiophen had an activity approximately one-third that of pethidine while 2-(3′-morpholino-1′-carbethoxy-1′-phenylpropyl)thiophen appeared to be four times as effective as pethidine. Other compounds prepared, such as 2-phenyl-2-diethylaminoethyl cyclohexanone were inactive.

F. H.

PHARMACOGNOSY

Cascara sagrada, Frangula and Oak Barks, Distinction between the Powders. G. G. d u C h a t e l i e r. (*Ann. pharm. franc.,* 1948, **6**, 507.) The powders of these three barks can be distinguished not only by the

presence or absence of stone cells but by the characters of the crystals in the sheaths surrounding the fibres. *Oak bark,* has prismatic crystals with numerous truncations with poorly defined edges, so that the crystals appear rounded. They are elongated with the long axis at right angles to the fibres. Monoclinic crystals (rhombs) very rare. *Cascara sagrada:* crystals generally square and smaller than those of oak bark. Truncations rare, or if present, sharply defined so that the crystals are more regular in shape than those of oak bark. Monoclinic crystals frequent and mostly square. *Frangula bark:* crystal forms intermediate betwen those of the other two powders. If the crystals in oak bark are slowly dissolved in hydrochloric acid, a detached lignified envelope can be seen within the pecto-cellulose compartment of the crystal sheath; this detached envelope cannot be seen in the other two barks when treated in like manner.   J. W. F.

PHARMACOLOGY AND THERAPEUTICS

Antabuse and Alcohol, Effect of on Respiration and Circulation. E. A s m u s s e n, J. H a l d, E. J a c o b s e n and G. J o r g e n s o n. (*Acta Pharmacol. Toxicol.,* 1948, **4**, 297.) Alcohol does not produce circulatory or respiratory symptoms in normal human beings in a dose equivalent to 20 g. of absolute alcohol but when experimental subjects were treated with antabuse 12 hours before the intake of alcohol there was a marked increase in ventilation, a decrease in alveolar carbon dioxide, an increased pulse rate, and a slight increase in cardiac output and oxygen consumption. Antabuse-prepared individuals under the influence of alcohol must form a substance that directly or indirectly increases the irritability of the respiratory centre, and the feeling of dyspnœa is due not to a bronchoconstriction but to this increased irritability. The comparatively slight increase in cardiac output shows that there is no serious risk of too heavy a load on the heart after clinical application of antabuse, even though patients complain of serious palpitation and subjective dyspnœa.   S. L. W.

Antabuse and Alcohol, Formation of Acetaldehyde after Ingestion of. J. H a l d and E. J a c o b s e n. (*Acta Pharmacol. Toxicol.,* 1948, **4**, 305.) After intake of alcohol, human subjects treated with antabuse show a much higher concentration of acetaldehyde in blood than do untreated individuals. The authors discuss the possibility that the symptoms observed after antabuse treatment may be explained as the result of this increased formation of acetaldehyde.   S. L. W.

Iodophthalein, Excretion from the Human Organism. H. O. B a n g and J. G e o r g. (*Acta Pharmacol. Toxicol.,* 1948, 4, 87.) A quantitative method for the determination of amcunts as small as 1 µg. of iodophthalein in fæces and organic fluids is described. Injections of iodophthalein, 500 mg. intravenously, were given to normal persons, and the concentrations in plasma and urine determined. After the injection a concentration of 10 to 20 mg. per cent. is obtained. This concentration falls steadily, reaching 2 or 3 mg. per cent. 24 hours after the injection. During the next few days iodophthalein is still demonstrable in the plasma and the concentration reaches zero by about the fifth day. Only small amounts of the substance are excreted in the urine. The great bulk of the substance is excreted in the fæces, the

LETTERS TO THE EDITOR

The Action of Decamethonium Iodide in Birds

Sir,—The mechanism by which neuromuscular block is produced in mammals varies with different substances. Drugs like d-tubocurarine chloride act solely on the motor end plate and render it insensitive to the depolarising action of acetylcholine. On the other hand decamethonium iodide can set up propagated contractions when applied sufficiently suddenly (Zaimis[1]) and, in any case, causes a depolarisation which extends to the motor end plate as well as to the muscle fibre (Paton and Vianna Dias[2]); it therefore produces neuromuscular block. Superficially, the paralysis produced in mammals by such an action is indistinguishable from that produced by the curarines. The position however is different when animals are used in which depolarisation of a muscle fibre produces not only electrical inexcitability but, in addition, contracture. This is the case with amphibian and avian muscle, as also with mammalian muscle after denervation. The contracture produced by decamethonium iodide on amphibian and denervated mammalian muscle has been described elsewhere (Paton and Zaimis[3], Zaimis[1]). The present experiments deal with avian muscle.

In adult fowls or chicks, and in pigeons, an intravenous injection of decamethonium iodide was found to cause a rigid extension of the limbs and retraction of the head (see Figure). If the dose is lethal the animal dies in this rigid condition, if the dose is below the lethal level the recovery is abrupt. Decamethonium iodide is highly active, 0·05 mg/kg. causing spasticity for about 3 minutes in chicks. This is a peripheral effect and the shortening of the muscle is probably a true contracture like that described by Langley as produced in the fowl by nicotine. The excitability of the muscle to nerve stimulation was found to be reduced. The full proof naturally requires electrical analysis of the condition. This reaction of avian muscle to decamethonium iodide is a further confirmation that the action is essentially like that of acetylcholine (Zaimis[1]). Tubocurarine chloride on the other hand causes the usual paralysis in birds (see Figure); a dose of 0·5 mg./kg. producing a paralysis that lasts for about 10 to 20 minutes in chicks. Succinoylcholine dibromide, another synthetic substance causing neuromuscular block (Bovet et al.[4], Walker[5]), and tetramethylammonium iodide which have both been found to depolarise muscle fibres in a similar way to decamethonium iodide (Paton and Vianna Dias[2]) produce the same spastic conditions when injected intravenously in birds. On the other hand tri-(diethylamino-ethoxy)-benzene triethyl iodide (Bovet et al.[6]), now called flaxedil, a synthetic substance, curare-like in most of its actions, causes in birds a paralysis like that of d-tubocurarine. From these findings it appears that not only amphibian muscle and denervated mammalian muscle but also avian muscle may be used as a test in differentiating a true " curare-like " blocking action from the superficially similar resultant action of substances like decamethonium iodide. The advantage of using the test on avian muscle is the ease with which the difference in the action of these two groups of drugs can be strikingly illustrated.

We are grateful to Dr. J. Walker for the supply of succinoylcholine dibromide. This work is being done with the aid of a grant from the Medical Research Council.

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November 1, 1949.

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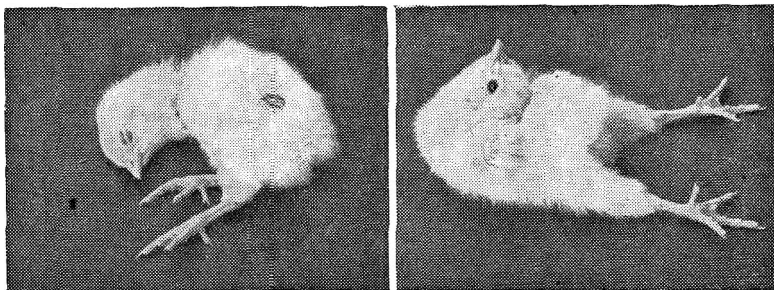
A comparison of the effects of an intravenous injection of decamethonium iodide (right) and d-tubocurarine chloride (left).

ABSTRACTS (continued from page 990)

excretion usually continuing for 8 to 10 days after the injection. A quantitative balance test was made on two persons and in both about 75 per cent. of the amount injected was recovered. The authors suggest that the balance is excreted in very small amounts (less than 1 mg. daily) over a period of several months after the injection. S. L. W.

Methyl Alcohol and Formic Acid Excretion in Man. A. L u n d. (Acta Pharmacol. Toxicol., 1948, **4,** 205.) After intake of small quantities of methyl alcohol (10 to 20 ml.) by human subjects no methyl alcohol was found in the blood in the course of 48 hours, and the concentration of formic acid in the urine was normal (6·5 to 12·8 mg. within 24 hours). Following intake of large amounts of methyl alcohol (50 ml.) this substance could be demonstrated in the blood (25 to 120 mg. per cent.) after 48 hours; formic acid could also be demonstrated in the blood (2·6 to 7·6 mg. per cent.) and in increased amounts in the urine (up to 2050 mg. per cent. within 24 hours). This increased excretion of formic acid after large methyl alcohol intake reaches its maximum in from 1 to ·3 days and is characteristic of methyl alcohol poisoning; cases of poisoning with methyl chloride do not show this increase. The author describes 5 fatal cases of methyl alcohol poisoning and gives figures of methyl alcohol and formic acid concentrations in blood and urine. S. L. W.

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