ALLETHRIN *

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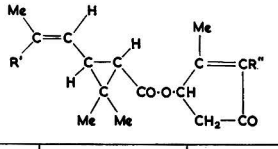
Synthetic work leading to the commercial preparation of allethrin {the ester of (±)-*cis-trans*-3-*iso*butenyl-2 : 2-dimethyl*cyclo*propane-1-carboxylic acid [(±)-*cis-trans*-chrysanthemic acid] with (±)-2-allyl-3-methyl*cyclo*pent-2-en-4-ol-1-one [(±)-allethrolone]} is reviewed briefly. The relative insecticidal activity and abundance of the eight isomeric esters present in allethrin are deduced and the most effective is shown to be (+)-allethronyl (+)-*trans*-chrysanthemate. The effect of various changes in the molecule of allethrin on insecticidal activity is described. The biological action of allethrin appears to be of a similar nature to that of the natural pyrethrins, and is associated with the particular stereochemical conformation in which the various parts of the molecule are held with respect to each other and with the chemical and physical properties of the groups so positioned. Although allethrin is inferior to the natural pyrethrins against most species of insects and by most methods of application, it is highly biologically active and, unlike many modern synthetic insecticides, has, so far, been found harmless to mammals.

ALLETHRIN is a synthetic compound closely related in structure to the four known insecticidal esters of the natural pyrethrins (Fig. 1). Methods were developed for the synthesis of the acidic and alcoholic components of the pyrethrins, to confirm structures for these compounds deduced from physical and degradative evidence and chemical reactions.[1-3] Allethrin is prepared by application of these routes. Therefore, it is proposed briefly to review synthetic work that led to the preparation of allethrin, then to discuss the properties of the eight isomeric forms that are present in the purified, commercial material, and finally to survey the structural features that have been found, by biological tests carried out at Rothamsted Experimental Station, to be essential in a molecule of this type if it is to have high insecticidal activity.



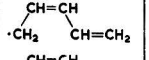
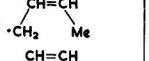
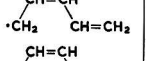
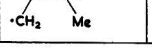
R'	R"	Name
Me		Pyrethrin I
Me		Cinerin I
MeO·CO		Pyrethrin II
MeO·CO		Cinerin II

FIG. 1

Many of the results were obtained in collaboration with Mr. P. H. Needham and Dr. C. Potter at Rothamsted. We have been very greatly assisted in our work by Dr. S. H. Harper & his co-workers in this country and by Dr. F. B. LaForge & his collaborators in America, who have been most generous in presenting to us compounds for bio-assay. Without their

* Read in a modified form before the Crop Protection Panel; for a report of the Discussion at the meeting, see *Chem. & Ind.*, 1954, p. 537.

help the programme of synthesis and insecticidal testing of compounds would, of necessity, have been much more limited in its scope.

Synthesis

Although earlier workers had attempted to determine the structure of the insecticidal material in the flower heads of *Chrysanthemum cinerariaefolium* and related species,[4] it was Staudinger & Ruzicka who laid the foundations of our present knowledge of their constitution. This work was carried out from 1910 to 1916 in Switzerland and published in 1924.[5] Although the structures assigned by Staudinger & Ruzicka to chrysanthemic and pyrethric acids were correct, it was not until 1947 that structures for pyrethrolone and cinerolone that fitted all the experimental evidence were deduced.[1–3] (For an exposition of the nomenclature of the pyrethrins and related compounds, see Harper.[21]) The structures of chrysanthemic acid and of cinerolone have been fully confirmed by synthesis.[5–12] Complete syntheses of naturally derived pyrethrolone and pyrethric acids have still to be carried out, but the structures shown in Fig. 1 for these compounds are based on sound evidence.[cf. 13] (The naturally derived geometrical isomer of pyrethrolone has been synthesized since this discussion.[13a])

No new methods of synthesizing acids and *cyclo*pentenones related to the pyrethrins were developed until 1945, when Campbell & Harper[6] carried out a practicable preparation of chrysanthemic acid, based on a route which, in the hands of Staudinger & Ruzicka,[5] had provided only sufficient of the required material for identification purposes (Fig. 2). Soon afterwards,

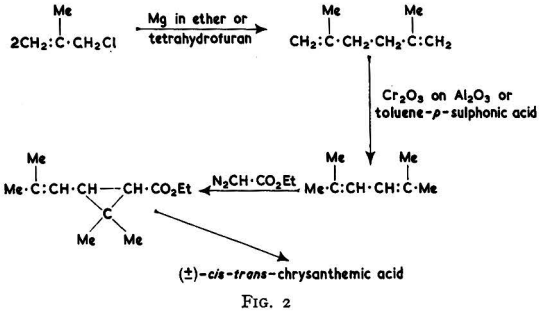


FIG. 2

Harper[14, 15] applied the cyclization of 2 : 5-diketones to the preparation of *cyclo*pentenones related to pyrethrolone and cinerolone (Fig. 3), and another route,[16] which also provides adequate yields of *cyclo*pentenones, is shown at the top of the Figure. When the side chain R in such ketones was saturated, it was possible to introduce bromine into position 4 of the ring;

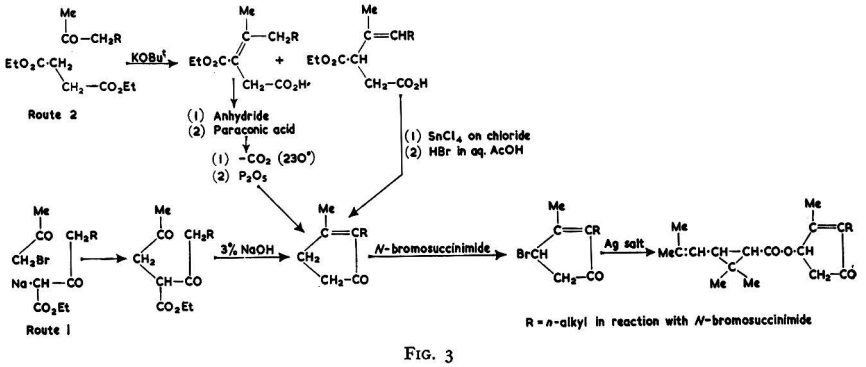


FIG. 3

by the treatment of such bromoketones with the silver salts of the chrysanthemic acids that Campbell & Harper had prepared, separated and resolved, Harper & his co-workers made dihydrocinerins I and tetrahydropyrethrins I.[15] If the acetates of the keto-alcohols were prepared by a similar method and then saponified with alcoholic potassium hydroxide, racemic dihydrocinerolone and tetrahydropyrethrolone, identical with naturally derived compounds, were obtained.[15; cf. 17, 18] If, however, the side chain R in the ketones was unsaturated, the reaction products with *N*-bromosuccinimide were tars,[15, 19] and the preparation of keto-alcohols with unsaturated side chains, such as are present in the naturally occurring *cyclo*pentenones, had to await the development of a route to 2 : 5-diketones in which a hydroxyl group was present before cyclization. Such a method was announced by Schechter, Green & LaForge[20] in 1949, and is shown in Fig. 4. Workers in the U.S.A.,[11, 20, 22, 23] in this country[9, 10, 13, 24, 25]

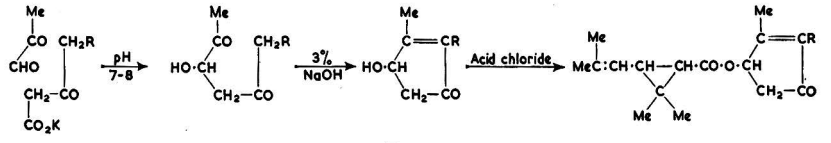


FIG. 4

and in Japan[26–28] have used this pyruvaldehyde route to make *cyclo*pentenolones with both saturated and unsaturated side chains. The logical application of this synthesis was to prepare keto-alcohols with a variety of unsaturated side chains from starting materials that were readily available; several practicable methods were already known for making the β-keto-esters required.[9, 10, 13, 14, 20, 24, 25, 29, 30] From the point of view of accessibility and cost of starting materials and the insecticidal effectiveness of esters of the keto-alcohols with synthetic chrysanthemic acid, the compound with an allyl side chain was found to be the best; when this alcohol is combined with (±)-*cis-trans*-chrysanthemic acid, the product is allethrin.

The manufacture of allethrin involves many stages; possible explosions from diazoacetic ester must be taken into consideration in the design of plant and pyruvaldehyde is an expensive intermediate. (Since this discussion took place, a detailed description of the manufacture of allethrin has been given by Sanders & Taft.[30a]) It is probably wise to regard allethrin as only the first synthetic material with biological properties related to those of the pyrethrins that has been produced.

Allethrin is a pale oil which can be distilled *in vacuo* and is said to be stable almost indefinitely when stored in the cold in the absence of oxygen, and to be appreciably more stable to heat, oxygen and ultra-violet light than are the natural pyrethrins.[31–36]

Stereochemistry: effect on biological properties

Allethrolone, the alcoholic component of allethrin, has no plane of symmetry and therefore dextro- and laevo-rotatory forms are possible. The asymmetric carbon atom is at C_4. An attempt is made in Fig. 5 to show the stereochemical difference between esters of (+)- and

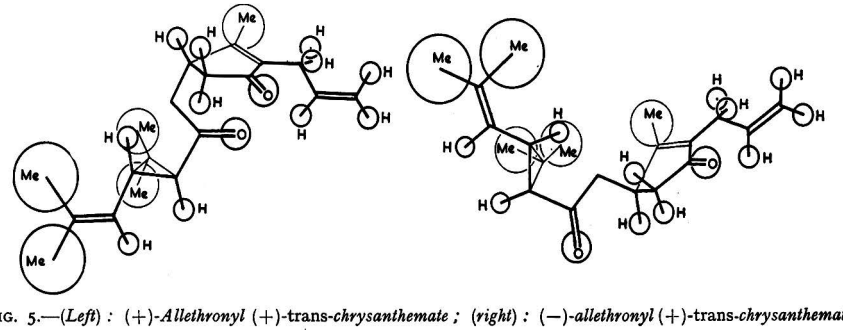


FIG. 5.—(*Left*): (+)-*Allethronyl* (+)-trans-*chrysanthemate*; (*right*): (−)-*allethronyl* (+)-trans-*chrysanthemate*

(—)-allethrolone with (+)-*trans*-chrysanthemic acid. (These, and subsequent Figures, are drawn neither accurately to scale nor in true perspective.) It is important to point out that, for the pyrethrins and allethrin, we have no idea of the conformation of the molecule when it exerts its biological action, since free rotation can occur at several bonds, especially those connecting the *cyclo*propane carboxyl group to the *cyclo*pentenolone ring. Therefore, the shape of the molecules represented in this Figure are chosen arbitrarily and do not necessarily bear much resemblance to those of the substances in action. In this case, the stereochemical arrangement adopted may depend on relatively weak, short-range, forces between the various groups in the molecule and on the interaction of these with the surface of the biological system where the insecticide acts. In this respect, allethrin and the pyrethrins differ from rotenone, the γ-isomer of benzene hexachloride, DDT, aldrin, dieldrin etc., which are far more rigid molecules.

Dr. LaForge has kindly given us samples of his resolved allethrolone, esterified with (+)- and (—)-*trans*-chrysanthemic acid,[37] and we find that the dextrorotatory isomer of the *cyclo*pentenolone gives esters that are about four times as toxic as those from the laevorotatory isomer. Similarly, (+)-cinerolone gives esters that are five times as toxic as those of (—)-cinerolone.[38, 42] The experimental evidence for this is shown in Table I. Gersdorff & Mitlin,[39] by an indirect method, appear to have deduced that (+)-allethrolone gives less toxic esters

Table I

Relative toxicities of esters from optical isomers of cinerolone and allethrolone

Alcohol	Chrysanthemic acid	*Phaedon cochleariae* Fab.	*Tenebrio molitor* L.
A (+)-*cis*-Cinerolone	(+)-*trans*	100	100
B (—)-*cis*-Cinerolone	(+)-*trans*	18	25
Mixture of A and B (1 : 1)		55	52
(+)-Allethrolone	(+)-*trans*	100	
(±)-Allethrolone	(+)-*trans*	64	
(—)-Allethrolone	(—)-*trans*	0·003	
(±)-Allethrolone	(±)-*trans*	22	

Measured drops in acetone [42]; cf. [38]

than (—)-allethrolone (they say: ' d-*trans* acid with d-allethrolone and with l-allethrolone have allethrin equivalents of 0·70 and 3·38 ' and ' . . . of the remaining two isomers, d-*trans*-acid with d-allethrolone and d-*trans* acid with l-allethrolone, one is 0·70 and the other 3·38 as toxic as allethrin '), and, by direct tests on houseflies have found esters of (—)-cinerolone a little less than twice as toxic as those of (+)-cinerolone.[38] This is the only instance in which the results of the American workers, with houseflies, are in wide disagreement with our results on various other insect species. Naturally derived pyrethrolone and cinerolone are both dextrorotatory and almost certainly of the same absolute configuration as each other. (+)-Allethrolone is probably in the same optical series and it is therefore most interesting that an unnatural optical isomer should be found more active than the naturally occurring one, with this particular insect species.

Chrysanthemic acid can exist in *cis*- and *trans*-forms; neither of these has a plane of symmetry and therefore a total of four isomers is possible. These are represented in Fig. 6. The assignments of optical configurations in this Figure are arbitrary and are not related to an optical standard by any conventional way of writing such formulae; an attempt has merely been made to indicate the stereochemical differences between the four forms. Campbell & Harper[6, 7] separated the four forms by fractional crystallization and optical resolution, and the relative toxicities of these four acids, esterified with the same keto-alcohol, to several species of insects are shown in Table II. The esters containing the laevorotatory acids are only one-fiftieth as toxic as those from the dextrorotatory forms and therefore contribute little to the effectiveness of the mixture. The esters from the (±)-*trans*-acid are about twice as toxic as those from the (±)-*cis*-form.

Crombie & Harper[40] have recently deduced the configurations of the optically active

chrysanthemic acids (3-*iso*but-1′-enyl-2 : 2-dimethyl*cyclo*propane-1-carboxylic acid) and have shown that (−)-*cis*-chrysanthemic acid is the epimer of (+)-*trans*-chrysanthemic acid at $C_{(1)}$. This means that (+)-*trans*- and (−)-*cis*-chrysanthemic acids have the same configuration at $C_{(3)}$. Since it is the (+)-*trans*- and (+)-*cis*-acids that give the more toxic esters, the implication is that it is the configuration at $C_{(1)}$ that is important for insecticidal activity.

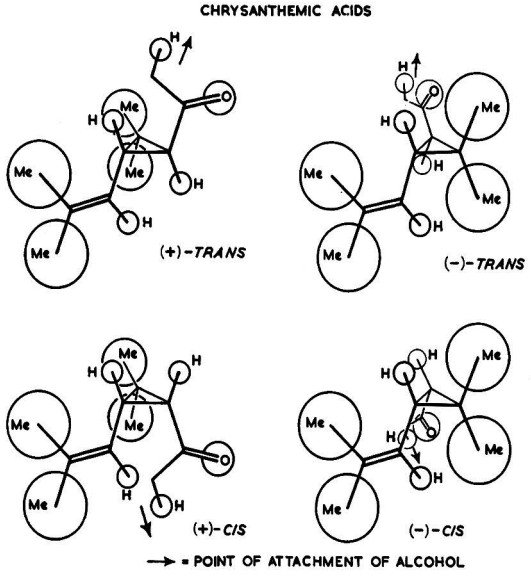


FIG. 6

Determination of proportions of isomers

It is of considerable interest and importance to know the relative proportions of the *cis*- and *trans*-isomers of chrysanthemic acid in the product from the addition of ethyl diazoacetate to tetramethylbutadiene. At the author's request, Dr. Harper and Dr. Crombie determined the proportions of the *cis*- and *trans*-acids in the mixture. The infra-red spectrum of (±)-*trans*-chrysanthemic acid or one of its esters shows bands that are not

Table II

Relative contact toxicities of esters of isomers of chrysanthemic acid

Alcohol side chain	Chrysanthemic acid	*Phaedon cochleariae* Fab. (*a*)	*Tenebrio molitor* L. (*a*)	*Dysdercus fasciatus* Sign. (*a*)	*Plutella maculipennis* Curt. (*b*)	*Macrosiphum euphorbiae* Thos. (*b*)	*Oryzaephilus surinamensis* L. (*b*)	*Musca domestica* L. (*c*)
(±)-Allyl	(+)-*trans*	100	100	100	100	100	100	100
,,	(±)-*trans*	35	56	53	72			64
,,	(+)-*cis*	21	63					
,,	(±)-*cis*	15	32	25	33			42
,,	(−)-*trans*	3			3			2
,,	(−)-*cis*	2	6					
,,	(±)-*cis-trans*	32	37		46	48	85	49
(±)-Methallyl	(+)-*trans*	63						
,,	(±)-*trans*	32						
,,	(±)-*cis*	18						

(*a*) Measured drops in acetone[42]
(*b*) Contact spray in 10% aqueous acetone[42]
(*c*) Campbell turntable[39]

present in that of an ester of (±)-*cis*-chrysanthemic acid, and *vice versa*, so that quantitative measurements on the esters of the three acids [the (±)-*trans*, the (±)-*cis* and (±)-*cis-trans*] give an estimate of the relative proportions of each in the mixture. This determination has been very kindly carried out by Dr. Crombie, who finds with the methyl esters that there is 68% of the *trans* and 32% of the *cis* in the synthetic mixture, and, with the specimen of allethrin we have used in our bio-assays, that it contains 75% of the *trans*- and 25% of the *cis*-acids.

Another obvious and, at first sight, reliable way of determining the relative proportions of the *cis*- and *trans*-acid isomers in allethrin would be to measure the relative toxicities, to several insect species, of the (±)-*trans*-, the (±)-*cis*- and the (±)-*cis-trans*-esters of the same alcohol, (±)-allethrolone. This biological method was applied by Gersdorff & Mitlin[39] to their results, which are shown, for comparison with ours, in Table II. Gersdorff & Mitlin conclude as follows: 'According to this bioassay, the *cis* isomers comprised about 69%, and the *trans* isomers about 31% of the sample of allethrin used in this study.' It must be emphasized that for such a method to be valid the constituents in the mixture must act independently of one another and that there must be a difference in toxicity of the two isomers. From tests by the American workers and from our results it is clear that both conditions are fulfilled. Here, then, is a most interesting discrepancy between the results of the chemical and biological methods of estimating this ratio.

The following argument may help, at least partly, to explain this difference between the results from the two methods, and should be borne in mind in conducting biological trials with all esters of this type. The (+)- and (−)-*trans*-chrysanthemic acids can be combined with (+)- and (−)-allethrolone in two ways, as shown in Table III, and rough relative potencies can be assigned to them from our knowledge of the toxicities of the separated forms. It is important to note that both contain equal proportions of the optical isomers and so no optical resolution without the aid of an asymmetric centre has been carried out; all that is suggested is that perhaps (+)-allethrolone may tend to combine more with (−)-*trans*-acid and (−)-allethrolone with (+)-*trans*-acid rather than the (+)-acid with the (+)-alcohol and the (−)-acid with the (−)-alcohol. These two forms, if obtainable, would be expected to have different insecticidal activities, as indicated. American workers[41] have found that if the (±)-*trans*-acid esterified with (±)-allethrolone is cooled, about 50% of it separates as a crystalline solid; this they have called the 'α-dl-*trans*' isomer; the remaining liquid is the 'β-dl-*trans*' isomer. The same crystalline isomer also separates from allethrin itself. Bio-assays by Gersdorff & Mitlin [39, 41] and at Rothamsted[42] have shown that the liquid 'β-dl-*trans*' isomer is considerably more toxic than the 'α' crystalline one. They must be constituted as shown in Table III.

Table III

Approximate relative toxicities of the isomeric forms of allethrin to a typical insect species

Acid	Alcohol	Potency	α- and β-Isomers
(+)-*trans*	(+)	100	50·3 'β-dl-*trans*'
(−)-*trans*	(−)	0·5	
(+)-*trans*	(−)	25	13·3 'α-dl-*trans*'
(−)-*trans*	(+)	2	
(+)-*cis*	(+)	48	24·1 'β-dl-*cis*'
(−)-*cis*	(−)	0·2	
(+)-*cis*	(−)	12	6·4 'α-dl-*cis*'
(−)-*cis*	(+)	0·8	

If the ratio of α- and β-forms in the (±)-*trans*-ester of (±)-allethrolone alone is not the same as it is in allethrin, then this method of estimation would be invalid, since the 'α- and β'-forms have different insecticidal activities. It is conceivable that such a variation in the relative proportions of α- and β-forms could be produced by different reaction conditions and varying, non-stoicheiometric ratios of the reagents in the preparation of the esters. Supporting this, it may be said that Gersdorff & Mitlin,[39] in the same paper in which the proportions of the *cis*- and *trans*-acids are determined as indicated above, estimate biologically

that 27% of the (±)-*trans*-fraction consisted of the 'α'-isomer (crystalline) and 73% of 'β'-isomer, yet about half the (±)-*trans*-ester actually separates as the crystalline isomer.[41] α- and β-Forms of the esters from the *cis*-acid must also be possible, although they have not been separated, and a similar variation in relative proportions may occur.

The factor rendering this biological method of estimating the relative proportions of *cis*- and *trans*-esters present in the mixture possibly open to question is that both acid and alcohol are in this case asymmetric and that esters of the optical isomers of both acid and alcohol have different insecticidal activities. The method would be unassailable with esters of (+)- or (−)-allethrolone or with esters of a symmetrical alcohol.

This argument is only a suggested explanation for the difference in the results by the two methods, and has not yet been tested experimentally. However, from the viewpoint of the manufacturers of allethrin it is unfortunate that the more toxic isomers present are diluted considerably with relatively ineffective material and that it is not practicable to separate the more potent isomers on a commercial scale. The relative toxicities of the four forms of allethrin containing the *trans*-acid have already been indicated, and the figures for the esters from the *cis*-acid are also shown in Table III. It is possible that such factors as these may be among those that account for the poor performance, to some insect species, of allethrin in comparison with the natural pyrethrins, in which Nature has carefully included only the most highly insecticidal isomers.

Toxicity of allethrin: effect of molecular changes

Saturation of the *iso*butenyl group in the acid to produce the ester (I) (Fig. 7) reduces the toxicity of the resultant esters by a factor of two or three.[42] But when the *cyclo*propane ring in (I) is replaced by a *trans*-double bond, to give (II), the resultant esters are without measurable toxicity.[42] This suggests that the *gem*-dimethyl group or the *cyclo*propane ring or both play an essential part in the toxicity of the compounds. Now it seems that this must be a stereochemical role, otherwise the esters of (+)- and (−)-*trans*-chrysanthemic acid would

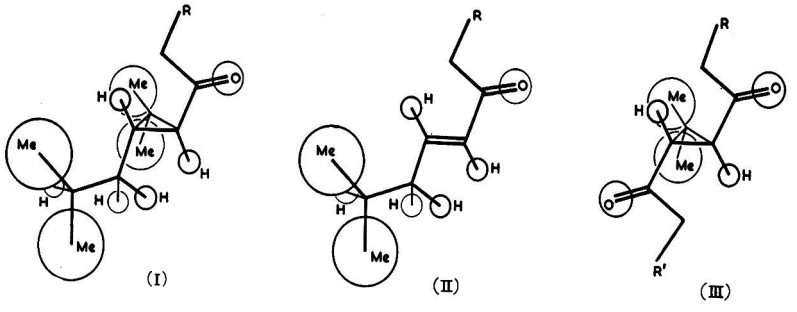


FIG. 7

not differ so widely in toxicity. When there is a great difference in biological activity between optically isomeric forms, the necessity for interaction of the active compound with its site of action at a minimum of three points is indicated. In the present system three of the groups involved appear to be the *iso*butenyl group in the acid, the *gem*-dimethyl group on the *cyclo*propane ring and the alcoholic part of the molecule. This is seen most clearly in Fig. 6. Thus if all groups in the ester of, for example, the (+)-*trans*-acid are in positions appropriate for highest biological activity, in the ester of the (−)-*trans*-acid, the *iso*butenyl group and the alkoxycarbonyl group can easily approach the surface of interaction, but the dimethyl groups on the *cyclo*propane ring are then remote from the location that was previously close to them. Additional evidence that the *iso*butenyl group is essential in the most active compounds is provided by almost complete lack of toxicity of the diallethronyl ester of (±)-*trans*-caronic acid[16, 42] [Fig. 7, (III), R = R′ = allethronyl] and of the allethronyl methyl ester of caronic acid [(III), R = Me, R′ = allethronyl]. In both these esters, either the (+)- or

(—)-form will fulfil all the requirements that were suggested above to be necessary for high activity, except that no *iso*butenyl group is present (but compare later remarks on changes in physical properties with each chemical change).

Some esters with various side chains are shown in Fig. 8. The difference in toxicity of the optical isomers of allethrolone (III) and cinerolone (VII) has already been discussed. A substituent at the 2-position of the *cyclo*pentenolone ring, other than hydrogen, is essential for high activity. Thus (I)[16] is very much less toxic than allethrin (III),[42] but with a *n*-butyl group in this position (II),[15] the compound is one-tenth as toxic as allethrin. At least one double bond in the side chain is necessary for highest toxicity, and its location is important. If the double bond is in the 3-position (IV)[24] the ester is about one-quarter as toxic as allethrin, and the compound (V),[25] with a pent-4′-enyl side chain, is not more toxic than that in which R′ is saturated (II). There is little detectable difference in activity between compounds with *cis*- and *trans*-crotyl side chains (VII)[9, 10] and (VIII)[24] and the *cis*-crotyl compound (VII; cinerin I) is perhaps slightly more toxic than allethrin (III). A methyl group on the end of the crotyl side chain (VII), producing (VI),[10] lowers the toxicity only slightly, but a 2′-methyl substituent on (III), giving (IX),[24] lowers the activity appreciably below that of (III), (VII) and (VIII). Therefore, for highest toxicity there should be a double bond in the 2′-position of the side chain, substituted, if at all, at the 3′-position only.

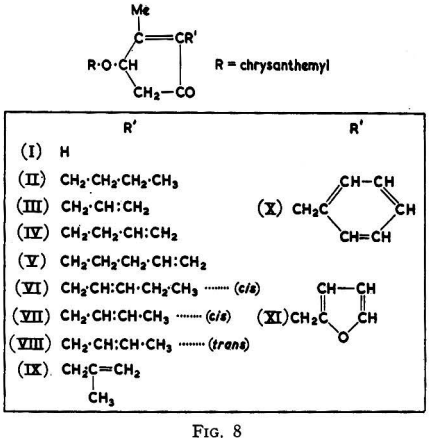


FIG. 8

Recently compounds (XI) (which is called Furethrin) and (X) have been kindly presented to us by workers at the Cooper Technical Bureau. Furethrin could be made on a large scale. In the limited tests we have so far carried out with *Phaedon cochleariae* (mustard beetles), we find no significant difference in toxicity of either from allethrin when differing molecular weights are taken into account.

Finally, we have investigated to a limited extent the biological effect of interfering with the keto group in allethrin[16, 42] (Fig. 9). The oxime of allethrin (IV) is considerably less toxic than the parent compound. When the keto group is reduced, the alcohol (III) produced has only one-tenth of the toxicity of the ketone to *Phaedon cochleariae* and to *Tenebrio molitor* adults.

Tribute should be paid to the foresight of Staudinger & Ruzicka, who made the mixture of compounds (I) and (II) shown in Fig. 9, and showed that they had some insecticidal activity, probably due to (I). At the time of their work, they were not aware that there was a double bond in the *cyclo*pentenone ring and that the acyloxy group was attached at the 4-position.

Thus, the insecticidal action of allethrin, as is the case with the natural pyrethrins,[43] appears to be associated with the particular stereochemical conformation in which the various parts of the molecule are held with respect to each other, and with the chemical and physical

properties of the groups so positioned. No esters of chrysanthemic acid, except those with alcohols closely related to pyrethrolone, cinerolone or allethrolone, have so far been reported to show characteristic pyrethrin-like activity, although other esters of the acids may well be found that have insecticidal activity of another type.[43] Similarly, the only acids that give insecticidal esters of the keto-alcohols are closely related to chrysanthemic acid.[43] When the

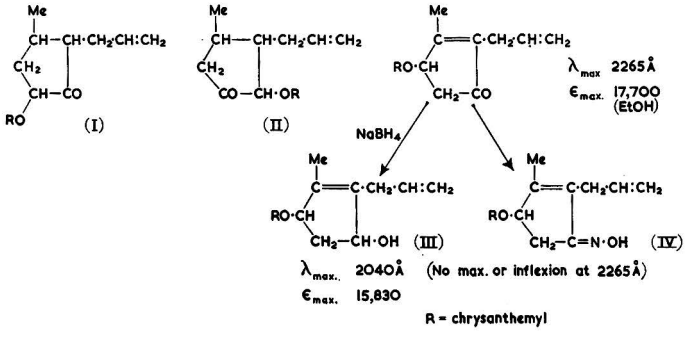


FIG. 9

natural pyrethrins mixture is compared with allethrin on a range of insect species by a direct spraying technique,[46] which applies the poisons as a spray and allows the insect to remain until inspection on a film of the insecticide, the relative toxicity is found to vary widely with the insect species examined.[44] Other workers, with different contact-poison techniques, have obtained similar results. However, with all the analogues of allethrin so far examined in our work, and by the limited range of application techniques used, the relative toxicity of any two compounds has been found remarkably constant, whatever the insect species examined. Again, if two modifications of the alcohol are each esterified with acid A and with acid B, the relative toxicity of the esters from the two alcohols is again constant and independent of the nature of the acid, provided that the compounds are toxic enough to be evaluated.

Such constancy of relative toxicities may be because a system common to all the insect species examined is attacked by these compounds, but a much more detailed knowledge of insect biochemistry is necessary before it can be known whether such a speculation has any real basis. In all studies of the effect of chemical changes on the biological activity of a range of related compounds, it must be constantly borne in mind that each alteration will affect the physical properties of the substance. It is conceivable that a compound may have the chemical and stereochemical requisites to make it a good insecticide, but such unfavourable physical characteristics that it cannot reach its site of action. It must be emphasized, however, that in all these biological results only the effect of poisons applied to the external surface of the insect has been estimated, and in most cases no residual film or injection techniques have been used. The two methods used have been the application of measured drops in acetone, to a specific location on the insect, a technique developed by Mr. P. H. Needham,[45] and spraying an aqueous emulsified suspension containing 10% of acetone in the tower designed by Dr. Potter.[46, 47] All the probit-regression lines obtained by both methods have been parallel for any insect in any one series of tests, which has simplified our comparisons of various compounds. Other workers, particularly in America, report that relative toxicities vary with the concentration applied, giving non-parallel probit-regression lines.

Allethrin is reported by workers who have examined the phenomenon to be inferior to the natural pyrethrins in rapid paralytic or ' knock-down ' effect. So far we have used death as the criterion for comparison of any two compounds, and it is not yet known whether knock-down is merely the first symptom of the action of the pyrethrins, of which death is a later manifestation, or whether knock-down and toxicity are independent of one another. We hope to extend our work to examine this and, by other methods of applying the insecticide, to try

to account for the poor performance of allethrin in comparison with the natural pyrethrins in some cases. However, it is no mean achievement to prepare synthetically any compound closely approaching in biological activity that of a natural product. Perhaps the property most in the favour of allethrin is that, unlike many synthetic insecticides in use today, it has so far been found quite harmless to mammals.

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THE FRACTIONATION OF THE NON-PROTEIN NITROGEN OF GRASSLAND HERBAGE

By W. S. FERGUSON and R. A. TERRY

The total non-protein nitrogenous (NPN) fraction of oven-dried grassland herbage has been examined. The NPN was obtained by extraction of dried herbage with boiling water and it was fractionated as follows: (1) precipitation of part of the peptide-nitrogen with 75% alcohol, followed by (2) precipitation of purines with silver sulphate (this treatment also precipitated some peptides), followed by (3) passage through a cation-exchange resin, which gave three main fractions: (*a*) non-adsorbed nitrogen compounds, including nitrate, pyrimidines and unidentified substances, (*b*) adsorbed compounds which were eluted with sodium hydroxide—amino-acids, amides, ammonia, betaines, peptides or 'bound' amino-acids, and unidentified substances and (*c*) basic compounds, including choline, and strongly adsorbed compounds that could not be eluted from the column with strong acid, probably mainly peptides and possibly including nucleotides.

Two grasses, a clover and a lucerne sample were used and the proportion of their total nitrogen present as NPN varied from 23 to 30%. An analysis including amino-nitrogen after hydrolysis, amide-nitrogen, ammonia-nitrogen, nitrate-nitrogen, purine-nitrogen, betaine-nitrogen and choline-nitrogen accounted for 77–88% of the NPN. The fractionation gave higher recoveries when assumptions were made on the probable peptide content and it indicated where unidentified substances might be found.

The non-protein nitrogenous (NPN) fraction of grassland herbage is a not inconsiderable fraction which can vary with the physiological state of the plants. In general, the more favourable the growth conditions the higher is the NPN as well as the total nitrogen content, and as growth progresses to maturity so the NPN content, expressed as a percentage of plant dry-matter, falls.

The major portion of the fraction consists of substances concerned in the synthesis of protein in the plant, and certain of these substances, particularly the amino-acids, have received the attention of several workers. Reference to this work has been made by Synge[1] and Bathurst.[2]

Purines and nucleosides, for the synthesis of nucleic acid, might be expected to be present in the fraction in relatively small amounts, and simple nitrogenous bases, the betaines and choline, are known to be present in most plant material. Finally, numerous substances, e.g. alkaloids, B-vitamins etc., will be present in trace amounts.

Although individual constituents and groups of compounds have received considerable attention, few workers have attempted to obtain an over-all picture of the make-up of the total NPN fraction of plants, particularly pasture plants. Vickery[3–8] made an intensive study of protein-free lucerne juice, but although he separated 17 fractions, by using conventional precipitation methods, the recovery of NPN was very incomplete.

Synge has used ionophoretic fractionations, a procedure that appears very valuable in effecting broad group separations of the NPN constituents, and Table I gives his suggested approximately quantitative distribution of NPN in the dialysable portion of rye-grass juice.

Table I

	Nitrogen, as % of total NPN
Non-amino-acid bases	15–25
Basic amino-acids, peptides etc.	6–15
γ-Aminobutyric acid	5–10
Glutamine	20–25
Other free neutral amino-acids	10–15
'Bound' amino-acids in neutral compounds	4–10
Free glutamic and aspartic acids	10–20
'Bound' amino-acids in acidic compounds	1–3

The present work was undertaken since it was thought that ion-exchange chromatography would offer a suitable means of separation of the NPN constituents. Experience has shown, however, that with complex mixtures the value of the method is limited. Mechanical blocking of columns, precipitation of purines in columns and almost irreversible adsorption occur when a protein-free pasture juice or aqueous extract is passed through an ion-exchange resin. It

has been found necessary to remove interfering substances by precipitation methods that must be suspect in quantitative work of this nature.

The procedure developed was: (1) hot-water extraction of oven-dried herbage, (2) precipitation of part of the peptides with 75% alcohol, (3) precipitation of purines with silver sulphate, (4) passing of the filtrate through a cation-exchange resin column to give three fractions: (*a*) not adsorbed, (*b*) adsorbed and eluted with alkali and (*c*) strongly adsorbed on resin and only partly removed by strong acid.

Analytical work on the fractions affords an approximate characterization of the NPN fraction. The betaines, choline, total NPN, amino-nitrogen, amide-nitrogen, ammonia-nitrogen and nitrate-nitrogen were determined on separate samples of the original dried herbage.

From the work of Bathurst & Allison[9] it was realized that oven drying of herbage could affect amides, cause proteolysis, and so increase the peptide fraction, but the development of analytical procedures and the identification of some of the minor nitrogenous constituents was the main object. Experience gained suggests that the examination might be simplified and improved by using separate alcoholic and aqueous extractions of fresh or freeze-dried materials.

Experimental methods

Analytical

Total nitrogen was determined in extracts and fractions by a micro-Kjeldahl method. Under ordinary laboratory conditions particular care is needed in this determination since when a very small proportion of the test solution is used errors are greatly magnified. For herbage samples a semi-macro Kjeldahl method was employed. When nitrate was present, digestion with acid was preceded by a reduction.

True-protein-nitrogen was determined on herbage samples that had been exhaustively extracted with boiling water after treatment with boiling 70% alcohol.[10] The alcohol treatment, it is considered, should render the protein insoluble. The total NPN was obtained by difference between total nitrogen and true-protein-nitrogen.

Amino-nitrogen was estimated by the Van Slyke manometric nitrous acid method, with a reaction time of 5 min. It is realized that certain amino-acids give high recoveries of amino-nitrogen by this method, that over 60% of the amide-nitrogen of glutamine is determined as amino-nitrogen, and that proline-nitrogen and the non-amino-nitrogen of the basic amino-acids are not determined, although under our conditions the recovery of amino-nitrogen in lysine was 80% of the total nitrogen. These errors are compensating to a degree dependent on the composition of the solution under test. Despite these shortcomings the Van Slyke method is probably the most convenient one available at the present time for routine work.

Ammonia when present in the test solution was removed by distillation under reduced pressure.

Ammonia and amides were determined in extracts by the methods developed by Vickery & co-workers.[10] In much of the work it was considered unnecessary to determine glutamine and asparagine separately and a figure for total amides only was obtained.

Nitrate-nitrogen was determined by the disulphonic acid method as described by Johnson & Ulrich,[11] *choline-nitrogen* by Engel's method[12] and *betaine-nitrogen* by the method developed by Cromwell & Rennie.[13]

Purine solutions, obtained as described below, were run on paper chromatograms, with butanol–ammonia as the solvent system. Five spots each of 5 μl. were run and the individual purine spots were detected under ultra-violet light. The paper was cut crosswise and the five spots of each purine were eluted from the paper with 5 ml. of 0·1N-hydrochloric acid.

Four separate spots were occasionally seen and, on being dissolved, their ultra-violet-absorption curves, between 230 and 300 mμ, agreed with those of adenine, guanine, xanthine and hypoxanthine. More usually, however, the quantity of hypoxanthine was small and therefore masked by the adenine spot. In this circumstance adenine and hypoxanthine were eluted together and determined as adenine.

Quantitative determination was made by comparison of absorption maxima of known amounts of purines after running and elution of paper chromatograms under uniform conditions.

Hydrolysis of solutions was carried out with 5N-hydrochloric acid at 100° for a minimum of 8 h. and the acid was removed by repeated distillation under reduced pressure. The humin-nitrogen formed varied between 1 and 10% of the nitrogen of the solution.

Extraction of non-protein nitrogenous fraction

Various extractions with cold and hot water were tried and the procedure finally adopted was to extract 100 g. of dried herbage with 4 l. of boiling water under reflux for 20 min. The mixture was allowed to cool before filtration. The filtrate usually measured about 3700 ml., but for subsequent calculations a recovery of 4000 ml. was assumed.

The nitrogen extracted in this way corresponded closely with that obtained in the exhaustive extraction by the quantitative method of Vickery.[10] The extract gave no precipitate with trichloroacetic acid, although occasionally a slight opalescence, and was assumed to be practically free of protein.

Precipitation of 'peptide' fraction with alcohol

The removal of the bulk of the peptides and polysaccharides from the aqueous extract was necessary for satisfactory running of resin columns. For this, alcohol precipitation was adopted, with concentration of 75% alcohol. A higher concentration of alcohol was not used since it was thought that nitrogenous compounds other than peptides might be lost.

A test was made on a grass extract with 70, 75, 80 and 85% alcohol and the quantities of nitrogen precipitated were 12·2, 15·3, 19·0 and 23·6% respectively of the total nitrogen. It might be expected that an alcohol concentration of between 80 and 85% would remove most of the non-dialysable peptides, and dialysis of the extract for a period of 7 days, with two changes of water, showed that 21·5% of the NPN did not pass through the Cellophane membrane. Dialysis of a redissolved precipitate obtained by an 85%-alcohol treatment, however, showed some 36% of the precipitated nitrogen to be dialysable.

The peptide fraction obtained by precipitation with 75% alcohol has not been examined in detail, but amino-nitrogen was determined before and after hydrolysis.

Removal of purines

After alcohol treatment of the aqueous extract, the precipitate was removed by filtration or centrifuging, and the alcohol in the filtrate distilled off under reduced pressure. The remaining solution, when passed through a column of Zeo-Karb 215, produced a band of insoluble material during elution with alkali. This resulted in severe 'tongues' at the alkali front and prevented a clear separation of eluted substances.

The insoluble material was found to contain purines, and methods for their removal were examined. Adsorption on charcoal, untreated charcoal and charcoal pretreated with phenol, copper salts and other compounds to reduce adsorptive power, was tried. Adsorption was efficient but the adsorbed substances could not be recovered on prolonged extraction with boiling methyl or ethyl alcohol, acetone, acid or alkali.

Precipitation of purines as silver salts was next tried. To avoid the introduction of nitrogenous compounds, silver sulphate was preferred as a precipitating agent to the more usual ammoniacal silver nitrate. Precipitation was carried out at pH 4 to minimize precipitation of basic amino-acids. The procedure was to add cold saturated silver sulphate solution to the extract, previously brought to pH 4 with sulphuric acid, until no further precipitate appeared. After standing overnight in the dark the bulky precipitate was centrifuged off and washed with acid at pH 4.

For the determination of purines the silver precipitate was decomposed with hydrogen sulphide. The solution was strongly coloured and contained a relatively high concentration of salts. For these reasons paper chromatograms streaked and gave no clear separation of the purines. It was necessary to reprecipitate the purines as silver salts and decompose them again with hydrogen sulphide to obtain a clean solution suitable for running on paper. The

individual purine spots were eluted from the paper for ultra-violet-absorption measurements.

The decomposition of the silver precipitate with hydrogen sulphide was very slow and complete recovery of nitrogen could not be attained. The precipitate was ground frequently during the hydrogen sulphide treatment and given at least six extractions. Decomposition with hydrochloric acid was tried, but recovery was no better and far more colour was extracted.

Separations on columns of Zeo-Karb 215

The solution obtained after centrifuging off the silver precipitate was passed without further treatment through a column of Zeo-Karb 215 that had been acid-treated and washed. The slight excess of silver it contained was held at the top of the column and did not interfere. The column was well washed with water. The solution and washings passing through were collected and examined for non-adsorbed nitrogenous compounds (*a*).

The column was then eluted with 0·1N-sodium hydroxide solution until some emerged unchanged. The eluate, containing amino-acids, amides etc., was examined further (*b*).

The column was then eluted with 2N-acid to remove strongly basic and adsorbed substances (*c*).

(*a*) *The acidic and non-adsorbed nitrogenous constituents.*—This fraction contained nitrate in quantities that agreed reasonably well with those found by direct determination on the original herbage. Little has been done to identify the other nitrogenous constituents, which in one grass sample accounted for some 12% of the total NPN.

(*b*) *Sodium hydroxide eluate of column.*—No quantitative determination of individual amino-acids has been attempted, but to obtain a picture of the amino-acids and amides present fractions were collected in some cases during elution and run on paper chromatograms with a butanol–acetic acid–water mixture. Other substances, such as the betaines, were sought in the fractions. In other samples the eluate was collected in one fraction for the determination of total, amino-, amide- and ammonia-nitrogen.

(*c*) *The strongly basic and adsorbed constituents.*—Prolonged elution of the column with 2N-hydrochloric acid removed only a small proportion of the nitrogenous substances. The nitrogen in the eluate, which was examined further, was probably not representative of the total adsorbed nitrogen.

Herbages examined

Four very different herbages have been used in this work to illustrate variations in the magnitude and composition of the NPN fraction.

(1) *Grass A.* This was a mixture of a perennial rye-grass and white clover in its fourth year. It had received 2 cwt. of superphosphate and 2 cwt. of Nitro-Chalk per acre in February, 1953, was grazed in the beginning of April, and was about 6 in. high and contained about 10% of clover when sampled on 19th May, 1953.

(2) *Grass B.* This consisted of almost pure leafy rye-grasses (S23 and S24) from irrigated plots receiving very high dressings of nitrogenous fertilizer—5 cwt. of Nitro-Chalk per acre after each cut; it was cut on 8th October, 1953, at a height of 8–10 in.

(3) S100 *white clover.* This was separated from a mixture of timothy, meadow fescue and white clover in its first harvest year and managed so as to retain a 50% clover sward. It had received 3 cwt. of superphosphate and 1 cwt. of potassium chloride in January, 1953, and was cut on 4th September, 1953, at a height of 8 in.

(4) *Lucerne.* This was a Du Puit lucerne drilled 20th July, 1953, in well-dunged land, with 4 cwt. of superphosphate and 2 cwt. of potassium chloride per acre in the seed-bed. It was cut on 9th October, 1953, at a height of 10–12 in.

The herbages were dried in shallow trays at 95° for 4 hours in an electric oven with a forced draught. The samples were then finely ground for analysis.

Results

Alcohol-precipitated 'peptide' fraction

The alcohol precipitates were examined for amino-nitrogen content before and after

hydrolysis. In these determinations ammonia was not removed and the amino-nitrogen value after hydrolysis therefore includes ammonia-nitrogen derived from any amides present in the peptide fraction. An appreciable quantity of ammonia-nitrogen was, in fact, found in the hydrolysed fraction of one grass sample. The figures obtained on four herbages, calculated as a percentage of dried herbage, are shown in Table II.

Table II

	Grass A	Grass B	Clover	Lucerne
Total nitrogen, mg.	58	102	130	188
Amino-nitrogen, mg.	4	8	27	59
Amino-nitrogen, mg., after hydrolysis (A)	37	53	101	155
(A) as % of total nitrogen	64	52	78	83

In the legumes some 80% of the nitrogen was accounted for as amino-nitrogen, after hydrolysis, whereas in the grasses the figures were 52 and 64%. The recoveries of amino-nitrogen in the grasses were unexpectedly low and further work on this is needed. From these rather inadequate results it might be assumed that the nitrogenous portion of the precipitate arising by treatment of the aqueous extract with 75% alcohol consists mainly of peptides.

A point of interest is the relative amounts of amino-nitrogen before and after hydrolysis in the grasses and legumes, suggesting peptides of shorter chain length in the legumes.

Silver-precipitated purine fraction

The treatment of a silver precipitate containing 100 mg. of nitrogen and obtained from a clover extract gave the results shown in Table III.

Table III

Repeated treatment with hydrogen sulphide in water suspension	
Nitrogen in residual silver sulphide, mg.	15·4
,, in solution, mg.	84·6
,, precipitated from solution on standing overnight, mg.	1·2
Solution containing 83·4 mg. of nitrogen reprecipitated with silver sulphate and centrifuged	
Nitrogen in precipitate, mg.	70·8
,, in centrifugate, mg.	12·6
Precipitate decomposed with hydrogen sulphide	
Nitrogen in first extract, mg.	60·6*
,, in second extract, mg.	1·3
,, in residual silver sulphide	2·3
Apparent loss, mg.	6·6

* Contained: Adenine-nitrogen 30·9 mg., guanine-nitrogen 16·5 mg., xanthine-nitrogen 9·3 mg., that is total purine-nitrogen 56·7 mg.; according to the ninhydrin colour test, no amino-acids were present.

In this particular clover extract about 60% of the nitrogen present in the silver precipitate was purine-nitrogen. In samples of grass A, grass B and lucerne the proportions were 32, 55 and 54% respectively. The recovery of purine-nitrogen was probably low since adsorption of purines on the silver sulphide seems likely.

Hydrolysis of a portion of the total silver precipitate with 5N-hydrochloric acid and subsequent running on a paper chromatogram showed the presence of a number of amino-acids. It is concluded, therefore, that some peptides are precipitated by the silver sulphate treatment, but quantitative results are lacking.

Fraction not adsorbed on Zeo-Karb 215

Nitrate was present in this fraction but no ninhydrin-reacting substances were detected.

Tests showed that pyrimidines were not adsorbed on Zeo-Karb 215 and could be identified on paper chromatograms by ultra-violet absorption. In a grass preparation the non-adsorbed fraction was examined and found to contain three substances that absorbed ultra-violet light. Two of these were probably thymine and uracil. The actual quantity of pyrimidine-nitrogen present in the herbages investigated has not been determined, but it appears unlikely that it would account for all of the unidentified nitrogen of the non-adsorbed fraction.

Sodium hydroxide eluate of Zeo-Karb 215

Amino-, amide- and ammonia-nitrogen accounted for about 70% of the total nitrogen in the eluate. Peptides, or 'bound' amino-acids, were present and, in one eluate from a grass extract, the amino-nitrogen was increased some 21% on acid hydrolysis. A considerable portion of the nitrogen in the eluate remains unaccounted for, but it must be remembered that the under-estimation of nitrogen in basic amino-acids and the omission of proline in the Van Slyke method would increase this portion. Without the quantitative determination of individual amino-acids in the eluate after hydrolysis it is impossible to assess the quantity of unknown nitrogenous substances present.

Fraction adsorbed on Zeo-Karb 215

Acid extracts of the columns were evaporated under reduced pressure several times to remove acid and it is possible that some hydrolysis of peptides occurred at this stage. Concentrated solutions were examined by paper chromatography, then hydrolysed with strong acid and again run on paper. Finally one sample was deaminated with nitrous acid and again run on paper. The general findings were that the unhydrolysed extracts gave a number of ninhydrin reacting spots—red and purple and yellow; their number was increased by hydrolysis and almost completely exhausted after deamination. There was definite evidence of the presence of substances that absorbed ultra-violet light at 260 mμ, presumed nucleosides, purines or pyrimidines. Insufficient material was available to state with certainty that nucleosides were hydrolysed to purines etc., but this seems likely.

Paper chromatograms treated with bismuth potassium iodide solution showed several spots, purple and brick red. One was due to choline but the others remain unidentified.

The nitrogen extracted from the column by acid is probably not representative of the total adsorbed nitrogen. It is suggested tentatively that the adsorbed substances consist largely of peptides, with some nucleosides and choline. This is supported by the figures in Table IV showing incomplete recovery of amino-nitrogen, before and after hydrolysis, from a column on sodium hydroxide elution.

Table IV

	Amino-nitrogen, mg.	
	Before hydrolysis	After hydrolysis
Put on column	34·1	46·2
Recovered in sodium hydroxide eluate	27·4	33·1
Left in column (by diff.)	6·7	13·1

In this particular case the figure of 13·1 mg. of amino-nitrogen after hydrolysis amounted to 78% of the adsorbed nitrogen.

Fractionation of herbage extracts

The results obtained on extracts of the four herbages by the procedure outlined are given in Table V. Choline and betaine values as determined on herbage samples are included in the appropriate fractions for completeness. Although amide- and ammonia-nitrogen were determined separately in the sodium hydroxide eluates of the grasses only their totals are quoted, since in another test some evidence was obtained of decomposition of glutamine and a consequent rise in ammonia-nitrogen.

It will be noted that the grasses and lucerne contained nitrogen equivalent to about 18% of crude protein (N × 6·25) and the clover nitrogen was higher, 26% of crude protein. The proportion of the total nitrogen extracted as NPN varied between 23·5 and 30·3%, the extremes being in grass A and lucerne respectively.

The fraction precipitated by alcohol, or presumed peptides, was small in grass A and large in the lucerne. The fraction accounted for 8·6 to 20·7% of the total NPN. If, however, the presumed peptides of the silver precipitate are added to those in the alcohol precipitate the peptide yields in the four species at this stage of the fractionation vary only between 20·4 and 24·2% of the NPN.

The purine contents of grass B and clover were equal, and appreciably higher than in the other samples. The purines accounted for 4·3 to 7·6% of the NPN.

Table V

Fractionation of non-protein nitrogen of herbages

	Nitrogen, mg./100 g. of herbage dry-matter				% of NPN			
	Grass A	Grass B	Clover	Lucerne	Grass A	Grass B	Clover	Lucerne
Total NPN	676	882	1022	862	—	—	—	—
Alcohol ppt., peptides	58	126	163	178	8·6	14·3	16·0	20·7
Silver ppt.	140	121	137	68	20·7	13·7	13·4	7·9
Containing purines*	45	67	67	37	6·7	7·6	6·6	4·3
Unidentified, probably peptides	95	54	70	31	14·0	6·1	6·8	3·6
Not adsorbed on column	80	176	96	78	11·8	19·9	9·4	9·0
Containing nitrate	60	71	40	37	8·8	8·0	3·9	4·3
Unidentified, including pyrimidines	20	105	56	41	3·0	11·9	5·5	4·7
Sodium hydroxide eluate of column	272	365	357	365	40·2	41·4	34·9	41·3
Containing amino	147	167	207	—	21·7	18·9	20·3	—
,, amide + ammonia	38	71	52	—	5·6	8·1	5·0	—
,, betaines	14	17	11	31	2·1	1·9	1·1	3·6
,, unidentified, including peptides	73	110	87	—	10·8	12·5	8·5	—
Adsorbed on column	126	94	269	182	18·7	10·7	26·3	21·1
Containing choline	14	16	8	4	2·1	1·8	0·8	0·5
Unidentified, including peptides, nucleotides	112	78	261	178	16·6	8·9	25·5	20·6
Total nitrogen in herbage dry-matter	2880	2996	4158	2842	—	—	—	—
NPN, as percentage of total nitrogen	23·5	29·4	24·6	30·3	—	—	—	—
* Containing: Adenine-nitrogen	30·2	41·2	36·2	30·4	4·5	4·7	3·5	3·5
Guanine-nitrogen	10·0	18·4	19·3	9·4	1·5	2·1	1·9	1·1
Xanthine-nitrogen	4·2	7·3	10·9	3·0	0·6	0·8	1·1	0·3

The fraction not adsorbed on the Zeo-Karb 215 column, consisting of acidic compounds and compounds not containing basic nitrogen groups, is an appreciable fraction of the NPN, particularly in grass B, an irrigated, heavily manured herbage. The nitrate-nitrogen values quoted are in three cases those obtained on the original herbage and not from the actual fraction. This is justified, as excellent recovery of nitrate-nitrogen was apparent in the remaining sample. The nitrate-nitrogen was 3·9–8·9% of the total NPN, and the remainder of the fraction, which includes a presumably small quantity of pyrimidine, 3·0–11·9%.

The nitrogen eluted from the column by sodium hydroxide was about 40% of the total NPN, the clover showed the lowest value of 35%. Analytical results on the constituents of this fraction are incomplete.

In the grasses and clover the amino-nitrogen was about 20% of the NPN. According to the work reported earlier on grass B some of this amino-nitrogen may have been derived from peptides, since the amino-nitrogen increased from 167 mg. to 202 mg. on hydrolysis. If the peptides are assumed to be dipeptides, the true free amino-acid-nitrogen would be reduced to 132 mg. or 15% of the NPN, and the peptide amino-nitrogen would amount to 70 mg., or 7·9%.

When the sodium hydroxide eluates were run on paper chromatograms and sprayed with ninhydrin the usual range of amino-acids was noted, alanine being the most plentiful throughout, with γ-aminobutyric acid the next in amount. Asparagine greatly exceeded glutamine in quantity. A number of unidentified spots were seen, presumably due to peptides.

The amide- plus ammonia-nitrogen values for the two grasses and the clover were 3·7, 5·4 and 5·0% of the NPN respectively. The recovery of this fraction from the original extracts was low. In the grasses the amide- plus ammonia-nitrogen contents of the solutions put on the column approximated closely to those determined on original extracts. It appears that amide, presumably in peptide form, was adsorbed on the column.

The nitrogen of the betaines represented only 1·1–3·6% of the NPN, the clover showing the lowest content and the lucerne the highest.

Figures for the unidentified portion of the sodium hydroxide eluate in the grasses and clover ranged from 8·5 to 12·5% of the NPN. In grass B, when allowance is made for peptide amino-nitrogen, the fraction is reduced from 12·5 to 8·5%.

The amount of nitrogen in the fractions adsorbed on the column, apart from the small quantity of choline-nitrogen, was large in grass A, clover and lucerne, being 16·6, 25·5 and 20·6% of the NPN respectively. In grass B the quantity was smaller at 8·9%. Although from one test mentioned above it would seem that a considerable part of this fraction consists of peptides it is unfortunate that difficulty in removing it from the resin prevented a direct examination.

It is evident that peptides are the most plentiful constituents of the total NPN and that they appear in most of the fractions separated—alcohol precipitate, silver precipitate, sodium hydroxide eluate of column and adsorbed on column. No doubt their distribution in the various fractions will depend on the nature and number of the amino-acids contained in the individual peptides.

Results of direct analyses of the herbages and NPN extracts are given in Table VI.

Table VI

Direct analyses of herbages and NPN extracts, mg./100 g. of herbage dry-matter

	Grass A	Grass B	Clover	Lucerne
Total nitrogen	2880	2998	4158	2842
Protein-nitrogen	2210	2101	3122	2016
N.P.N.	670	897	1036	826
Nitrate-nitrogen	60	71	40	37
Ammonia-nitrogen	17	29	27	17
Amide-nitrogen	31	87	135	75
Amino-nitrogen	175	242	345	311
Amino-nitrogen after hydrolysis	314	386	478	488
Humin-nitrogen after hydrolysis	25	32	38	38

The values for amino-nitrogen before hydrolysis do not represent the free amino-acid-nitrogen, since terminal groups of the peptides will be included. The true values for free amino-acid-nitrogen are more nearly those shown in Table V, in the sodium hydroxide eluate of the column.

From all the results collected the approximate composition of the NPN can be assessed. The figures are given in Table VII.

Table VII

Composition of NPN of herbages by direct analyses, % of NPN

	Grass A	Grass B	Clover	Lucerne
Amino-nitrogen after hydrolysis and humin-nitrogen (free amino-acid-nitrogen and peptide amino-nitrogen)	50·6	46·6	49·8	63·7
Amide-nitrogen	4·6	9·7	13·0	9·1
Ammonia-nitrogen	2·5	3·2	2·6	2·1
Nitrate-nitrogen	9·0	7·9	3·9	4·5
Purine-nitrogen	6·7	7·5	6·7	4·5
Betaine-nitrogen	2·1	1·9	1·0	3·7
Choline-nitrogen	2·1	1·8	0·8	0·5
Total	77·6	78·6	77·8	88·1

The free amino- + peptide-nitrogen account for 46–63% of the NPN, and from Table V it can be assumed that the free amino-nitrogen in the grasses and clover did not exceed about 20% of the NPN.

The amide-nitrogen varied widely in the different samples. It is assumed that both free and peptide amide-nitrogen are included in the values unless the peptide amides are not hydrolysed by the acid used in the determination.

The recovery of NPN was about 78% in the grasses and clover, but 88% in the lucerne. It must be remembered that the figures for amino-nitrogen may be appreciably low owing to

the deficiencies of the Van Slyke method of determination, and that the purines were probably underestimated.

In the main fractionations, fractions were obtained that were presumed to consist largely of peptides. It is interesting to see how these results (Table VIII) differ from those given in Table VII.

Table VIII

Composition of NPN of herbages by fractionation, %

	Grass A	Grass B	Clover
Mainly peptides			
Alcohol precipitate	8·6	14·3	16·0
Silver precipitate less purine-nitrogen	14·0	6·1	6·8
Adsorbed on column, less choline-nitrogen	16·6	8·9	25·5
Eluted from column	9·2	7·9	8·4
	48·4	37·2	56·7
Amino-acid-nitrogen	17·2	15·0	16·0
Amide- + ammonia-nitrogen	5·6	8·1	5·0
Nitrate-nitrogen	8·8	8·0	3·9
Purine-, choline- and betaine-nitrogen	10·9	11·3	8·5
Total	90·9	79·6	90·1
Nitrogen not accounted for on column	6·1	8·5	4·4
Nitrogen not adsorbed on column less nitrate-nitrogen, includes pyrimidines	3·0	11·9	5·5

In this it has been assumed that the peptides in the sodium hydroxide eluate of the column were dipeptides, also since no hydrolyses of the eluates of grass A and clover were made it is assumed that they contained the same proportion of amino- and peptide amino-nitrogen as grass B.

In grass B the sum of the figures for peptide- and amino-nitrogen is almost identical with that in Table VII when allowance is made for the amide-nitrogen present—the difference between the figures for amide- + ammonia-nitrogen in the two Tables. In the other grass and the clover the figures are appreciably higher. These peptide-nitrogen values would be expected to be above the true values, but they may be closer to them than the values obtained by difference between amino-nitrogen before and after hydrolysis.

It is of interest to note the variation in the quantity of presumed peptides in the four fractions, and in particular the low quantity of adsorbed peptide in grass B and the high amount in the clover, which might indicate peptides containing basic amino-acids.

The nitrogen of unknown composition not accounted for as presumed peptides and other determined compounds ranged from 9 to 20% of the NPN, part, which includes some pyrimidines, not being adsorbed on the column and the remainder in the sodium hydroxide eluate of the column.

Discussion

The presence of peptides at practically every stage of the fractionation described has greatly complicated the work. As the range of peptides is likely to be wide it is perhaps too much to expect their complete removal by any single physical technique. Synge, by working on dialysed juice, greatly reduced the interference by peptides. The peptides, however, form the largest fraction of the NPN and it is desirable to know more about their magnitude and nature.

It was hoped that 75% alcohol would precipitate the bulk of the peptides from NPN solutions, but results showed that only a relatively small proportion could be so eliminated in certain herbage samples, also that the peptide chain-length of this fraction, as inferred from amino-nitrogen figures before and after hydrolysis, varied widely in the different herbage species.

It may be that the amino-acid composition of the peptides determines their fate in the fractionation and that a useful separation is attained, at least, by alcohol and silver precipitations. Obviously, however, the further fractionation by the use of Zeo-Karb 215 columns cannot be recommended unless a means is found of recovering the strongly adsorbed substances. It is possible that columns of other materials would be more satisfactory.

Bathurst & Allison have shown the changes arising in the nitrogenous constituents during the drying of herbage samples. Apart from changes in the proportions of the acid amides and ammonia the total quantity of nitrogen extracted by 80% alcohol or water is greatly increased by oven-drying, an increase due to presumed proteolysis. By using a freeze-drying technique, proteolysis apparently does not occur to any marked degree, and the changes in amides are greatly reduced. It seems essential, therefore, that, in investigations on the NPN fraction, when it is not possible to use fresh herbage the material should be efficiently freeze-dried. Had such material been used in the present work the total quantities of peptides would have been smaller, but their separation not necessarily easier.

Bathurst concludes that 80%-alcohol extracts of freeze-dried herbages are virtually free of peptides. If this is true generally, separate detailed examinations of alcoholic and aqueous extracts might be preferable to a single examination of an aqueous extract, although the problem of separating the various peptides would remain.

For critical work on the simple constituents and low-molecular-weight peptides Synge's technique of ionophoretic separation with fresh herbage juices warrants further study.

In the present work the fractionation has yielded a broad picture of the composition of the NPN fraction of the oven-dried herbages examined, and has indicated where some of the unknown constituents are to be found. Of the less commonly known constituents the purines are present in substantial amounts, pyrimidines were found and the presence of nucleosides or nucleotides was indicated.

It is of interest to note that de Man[14] isolated 50 mg. of adenine, containing 26 mg. of nitrogen, from an aqueous extract of 100 g. of dried perennial rye-grass, a quantity similar to those determined in the present work. de Man looked for other purines, but failed to isolate any. He found a small quantity of the pyrimidine, cytosine, in a hydrolysed extract and concluded it was present in a combined form as a nucleoside or nucleotide.

In view of the exploratory nature of the work, the few herbage samples examined and their drying treatments it is unwise to compare the results. It is of interest to note, however, that in grass B—autumn grass, irrigated and heavily manured—the presumed peptides expressed as a percentage of the NPN was appreciably lower than those in grass A—a June grass, normal manuring. Expressed on herbage dry-matter, however, the yields of peptides are very similar in both grasses, the extra NPN in grass B being mainly in an unidentified fraction, possibly acidic, which accompanies nitrate in the fractionation.

The clover contained some 50% more NPN than grass A, but the composition of the NPN was similar although the clover contained less nitrate- and appreciably more amide-nitrogen, apparently in peptide form.

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THE CHEMICAL CONSTITUENTS OF VICTORIA PLUMS: PRELIMINARY QUALITATIVE ANALYSIS

By DENIS DICKINSON and JOY H. GAWLER

Victoria plums from a number of sources, and including samples from trees on different rootstocks, have been examined with a view to identifying their major and minor constituents. The red pigment common to all samples is cyanidin-3-monoglucoside, and obvious differences in colour appear to be due to variations in the amounts of green or brown pigments. The present paper concerns the essentially qualitative preliminary examination of the fruits, which is to be followed by quantitative analyses continued over several years.

Introduction

A recent paper by Parkinson[1] has explained the possible importance of the minor chemical constituents of the Victoria plum on the course of the reactions involved in the corrosion of the tinplate container by the canned fruit. These reactions, which are subject to spasmodic acceleration by unidentified substances, are of considerable economic importance to the fruit-canning industry of the United Kingdom.

The object of our investigation is to search for differences between the raw fruits that might occur through causes associated with different fruit-growing areas, rootstock or seasons. That there are differences between lots of Victoria plums passing through the fruit market is obvious from a cursory inspection of the ripe fruit; some are predominantly red, some are basically golden flushed with red, and other lots are more brown than red. It is planned to continue the investigation for a number of years, obtaining fruits from the same sources each year, and to carry out qualitative and quantitative analyses, paying particular attention to the minor constituents.

Parkinson has shown by various means that the natural colouring matter present in canned Victoria plums is chrysanthemin (cyanidin-3-monoglucoside), that the major acid constituent is malic acid, and that chlorogenic acid and quinic acid are also present. We have confirmed these findings in fruits obtained from the main growing areas and have added several other substances to the list. Quinic acid does not appear to be present invariably and there are already indications of considerable quantitative variation in several of the minor constituents.

Experimental

Sampling

Samples of authentic Victoria plums at least 12 lb. in weight were obtained from several growers in areas in Kent, Gloucestershire and Lincolnshire. Distinction was made where possible between trees growing on the three rootstocks Common Plum, Myrobalan and Pershore. For this work, sufficient plums for the purpose were taken from each sample, being selected as fully ripe on the grounds of appearance and texture. Damaged, bruised and discoloured fruits were avoided. Of each sample, a suitable proportion was preserved by bottling in water, and in some instances fruits were preserved by quick-freezing and storing at 0° F in case further plums should be required at a later date.

Preparation of extracts

Concentrated extracts were prepared from the fruits within several hours of receipt of the samples. The general method of preparation followed was:

The fruit or the skins were boiled for about five minutes in approximately 1% hydrochloric acid solution, such mild treatment being just sufficient to secure extraction of the anthocyanins and not sufficient to effect appreciable hydrolysis and removal of the sugar residues, or to attack any leuco-compounds that might be present.[2] Propanol was then added to the cool solution with continuous stirring until pectin was precipitated in a more or less granular form—as distinct from the gelatinous form which first appears—and the solution was then filtered. The filtrate was saturated with salt, causing separation into two layers and simultaneous salting-out of the anthocyanin from the aqueous layer. The alcoholic layer was separated and concentrated slowly

on a water bath exposed to a good current of air. The extracts prepared are described in Table I.

Table I

Details of extracts of Victoria plums examined

Extract no.	Origin of fruit		Fruit or skin
	County	Rootstock	
1	Gloucestershire	Pershore	Fruit
2	,,	Myrobalan	,,
3	,,	Common Plum	,,
4	Kent	Myrobalan	Skins
5	,,	Common Plum	,,
6	Gloucestershire	Myrobalan	,,
7	,,	Myrobalan	,,
8	Lincolnshire	Unknown	,,

Identification of constituents

The extracts were examined by paper chromatography. From preliminary trials it was found that satisfactory separation of the constituents and reproducibility of results required (*a*) the use of butanol–water–acetic acid (63 : 27 : 10) as solvent ; (*b*) preliminary washing of the paper with this solvent followed by atmospheric drying ; (*c*) equilibration of the paper with the solvent before use ; (*d*) the use of the descending technique in a tank. Temperature control was not found to be necessary, probably because marked fluctuation in the room temperature did not occur during the runs. The ' chromatocoil ' technique[3] was tried extensively, but, although it proved useful for the comparison of the suitability of solvents, it was not found generally satisfactory owing to the tendency of substances to migrate to one edge of the paper strip and to form bands instead of discrete spots.

Preliminary examination of the papers in visible light revealed the separation of a red anthocyanin pigment ($R_F \sim 0{\cdot}31$) and of a material of variable colour—green to brown—in the region of R_F 0·95. In ultra-violet light several other spots appeared, two of which, with R_F 0·62 and 0·87, became visibly yellow after treatment with ammonia vapour.

(i) *The anthocyanin.*—The position of the anthocyanin spot on the paper indicated that it might be a cyanidin-glucoside.[4] A sample of chrysanthemin (cyanidin-3-monoglucoside) was prepared from elderberries,[5] use being made of the tests of Robinson & Robinson[6] to check its identity, and when this was run in a similar manner alongside one of the plum extracts, identical spots were obtained, each with R_F 0·37. (The identity of the chrysanthemin has been further checked spectrographically, through the courtesy of Mr. T. L. Parkinson.) The identity of the pigments in the extracts from the two fruits was confirmed by purification and hydrolysis of the pigments, isolation of the plum pigment proving possible by adsorption on an alumina column followed by elution with 1% malic acid solution, as described by Parkinson.[1] Again by following Parkinson's technique, the hydrolysis products were found to correspond to cyanidin and glucose.

All the Victoria plums contained cyanidin-3-monoglucoside as the chief colouring matter.

(ii) *Chlorogenic acid.*—The substance that gave a blue fluorescent spot (R_F 0·61) was identified as chlorogenic acid by comparing its behaviour on paper with authentic samples of the substance, and also by separating it from the extracts and noting its reaction in solution.

When run on paper in parallel with authentic chlorogenic acid, the same R_F value was obtained (0·61). Narrow strips were then cut from the paper (in the transverse direction) so that each strip contained a portion of both ' known ' and ' unknown ' spots. The reactions of both portions when sprayed with potassium permanganate, ferric chloride and diazotized benzidine[7] were identical. A spray of 1% sodium nitrite in 10% acetic acid followed by N-potassium hydroxide solution produced the characteristic colour reaction—yellow changing to red—of chlorogenic acid.[8]

Diluted plum extract was placed on a column of filter-paper pulp and allowed to drain. This left two visible bands, one red, one brown, on the column. The brown band was eluted with ethyl acetate, and the solution obtained was passed through an alumina column. A

distinct yellow band now separated, and this was eluted by 1% malic acid solution to give a yellow solution. This material showed a strong blue fluorescence in ultra-violet light, changing to duck-egg green in ammonia vapour. It gave the characteristic colour change with sodium nitrite reagent and, when run on paper with butanol–acetic acid as solvent, it gave a single spot identical in position and reactions with that of chlorogenic acid.

(iii) *Isomeride of chlorogenic acid.*—The occurrence of a second spot at the position R_F 0·87, giving reactions identical with those of chlorogenic acid, leads to the supposition that an isomer of chlorogenic acid occurs in these extracts. The quantity of this isomer present appears to be greater than that of chlorogenic acid itself. In addition to its reactions, this substance when extracted from paper and hydrolysed gave the same hydrolysis products as *iso*chlorogenic and chlorogenic acids. In butanol–acetic acid–water its R_F value is considerably higher than those of chlorogenic, *iso*chlorogenic and *neo*chlorogenic acids, which were run in parallel with it. Further work on the isolation and characterization of this substance is proceeding.

(iv) *Malic, phosphoric and quinic acids.*—Parkinson[1] reported the occurrence of malic and quinic acids in canned Victoria plums, and their presence in extracts of the fresh fruits has been confirmed. Separation of the acids was effected by diluting the extract with alcohol and passing the solution through a column of De-Acidite E. The column was washed through with N-sodium carbonate solution and the eluate was freed from alkali by passing it through Zeo-Karb 216. The eluted solution of the acids was concentrated and spotted on paper along with solutions of malic, quinic, tartaric, citric and benzoic acids, and caused to run with butanol–acetic acid solvent. Indicator sprays of bromocresol green and bromophenol blue were used with some success, but better results were obtained with glucose, ammoniacal silver nitrate and sodium thiosulphate, according to the instructions of Anet & Reynolds.[9]

Because of difficulties in developing acid spots, the opportunity was taken of improving the technique when it was found that a number of acids could be shown up well by using a dual spray of ferric chloride and ammonium thiocyanate. The solutions recommended are: (*a*) ferric chloride (0·2%) in 70% industrial spirit, acidified by the addition of a few drops of 0·1N-hydrochloric acid per 100 ml.; (*b*) ammonium thiocyanate (0·5%) in 70% industrial spirit.

Table II

Reactions of some acids to the ferric chloride/ammonium thiocyanate spray

Acid	Colour of spot	Quantity detected, μg.	Remarks
Ascorbic	White	2·5	
Citric	,,	5·0	Develops as paper dries
Malic	Pale yellow turning white	12·0	,,
Quinic	Pale yellow	25·0	
Tartaric	,,	25·0	
Lactic	,,	25·0	
Aminoacetic	White	5·0	
Glutamic	,,	5·0	
Oxalic	Yellow	25·0	
Phosphoric	White	25·0	

The method of application is as follows: after a preliminary drying of the paper, spray lightly with the iron solution, then overspray with the thiocyanate solution. The acids may then be located approximately as white or yellowish spots on a speckled red background. Finally, spray again with the iron solution, thus intensifying the background to an even red-brown colour and delineating the acid spots.

Benzoic, maleic, succinic, *iso*butyric, adipic, trichloroacetic and chlorogenic acids (in addition to acetic acid in the solvent) gave no reaction. Certain acids that are detectable in this way, their reactions, and the approximate limits of quantity that may be easily detected, are given in Table II.

There was no interference from residual acetic acid in the paper, and in fact an over-all acid reaction is an advantage. It is essential to wash through the paper with 2N-acetic acid before use.

The plum extracts gave acid spots corresponding to malic acid (R_F 0·53) and quinic acid (R_F 0·26). The concentration of quinic acid appeared to be higher in the skins of the fruit. A third acid spot occurred with R_F 0·13, which corresponds with phosphoric acid. The spots in this position were all tested with a solution of ammonium molybdate in dilute nitric acid, followed by amidol reagent. In all cases the colour changes, first yellow, then blue, indicated phosphoric acid to be present.

Of the various extracts (Table I), all contained malic and phosphoric acids, although the reaction for phosphoric acid in extract no. 3 indicated only a faint trace. Quinic acid was not detected in extract no. 6.

In order to discount the possibility of acids (especially quinic) being produced in the extracts during storage—all the extracts were by now about four months old—a new extract was prepared from some of the Victoria plums that had been frozen at the time of harvest and held at 0° F. This new extract also contained malic, phosphoric and quinic acids. The identity of the anthocyanin was also confirmed in this new extract.

(v) *Sugars.*—The juices from the bottled plums were first used for the study on sugars. The fruits were bottled on harvesting, by a normal standard technique, and the bottles were stored in the dark at 2° C (± 1°) until required for examination several months later. The sugars likely to be present were thought to be glucose, fructose, rhamnose, galactose and sucrose.

In preparation for examination the juice was strained off from the fruit and acetone was added to precipitate the pectin. This was removed by filtration and the acetone was then distilled off from a water bath. The aqueous solution remaining was passed through a column of De-Acidite E to remove the acids, and then through alumina to remove the colouring matters and any remaining chlorogenic acid. The solution was then spotted on paper and run with butanol–water–acetic acid with appropriate markers, and only one spot was found. This corresponded to glucose, but inclusion of fructose within it was suspected, and galactose remained a possibility. A trace of sucrose was found in one bottle. Further separation of the sugars was then effected with ethyl acetate–acetic acid–water (3 : 1 : 3) as solvent, and by allowing the solvent to over-run the paper.[10] In this way distinct spots corresponding to glucose and fructose were revealed on spraying with ammoniacal silver nitrate. Confirmation of the presence of fructose was given by a positive reaction in solution with Folin–Denis reagent,[11] and by a further run on paper with butanol–acetic acid, spraying with α-naphthol/phosphoric acid.

The sugar solution was tested quantitatively for reducing power both before and after hydrolysis; as a result, disaccharides were concluded to be absent.

Since any sucrose contained in plums might possibly undergo complete inversion during the bottling process, a sample of frozen fruit was examined for sucrose.

The juice obtained by thawing and pressing the fruit was treated with acetone to precipitate pectin, which was then filtered off. The solution was passed through a column of De-Acidite E to remove acids and through an alumina column to remove colouring matters and any remaining chlorogenic acid. The solution was spotted on filter paper with fructose, glucose and sucrose as markers, and was run with ethyl acetate–acetic acid–water (3 : 1 : 3). Glucose and fructose were located by spraying with ammoniacal silver nitrate, and sucrose by spraying with α-naphthol/phosphoric acid. The solution gave spots corresponding to glucose, fructose and sucrose.

(vi) *Other constituents.*—Among several other substances that so far remain unidentified are the brown-green colours that separated from the propanol extracts when run on paper (R_F 0·95 in butanol–water–acetic acid). A small quantity of extract was diluted with a mixture of propanol and water and passed through a column of paper pulp. The anthocyanin and the chlorogenic acid were eluted with 1% malic acid solution, leaving a brown band undisturbed. This was subsequently collected in acetone.

It was suspected that the brown-green materials were derived from chlorophyll and might either be present in the original fruit, or be formed from chlorophyll during the extraction process. An extract of laurel leaves was prepared in aqueous alcohol, heated with dilute hydrochloric acid

and concentrated. The resultant brown solution was extracted with butanol and salt, separated, and the alcoholic solution concentrated on the water bath, thus following a procedure closely similar to that used for the plums. This solution, the acetone solution of the brown materials separated from plum extract, and extract no. 6 were all spotted on paper and run in parallel in butanol–water–acetic acid. All three gave brown spots of R_F 0·96.

This is taken as indicating that chlorophyll or its degradation products remain in certain ripe Victoria plums, and, since variations in the quantities of substances of this nature may well account for obvious differences in colour between fruits, the matter is to be investigated further.

Summary and conclusions

Using predominantly the techniques of paper chromatography, supplemented by spot tests, it has been shown that the colouring matter in ripe Victoria plums is cyanidin-3-monoglucoside and that this appears to be the only anthocyanin present. There appear to be varying amounts of chlorophyll or its degradation products in plums from different areas, but this requires further investigation.

Chlorogenic acid occurs in Victoria plums, as also do malic and phosphoric acids. Quinic acid was not found in all samples. Citric, tartaric and other fruit acids are absent and no trace of caffeic acid could be detected.

The sugars in Victoria plums are fructose, glucose and sucrose. Rhamnose and galactose could not be detected and sucrose was not always detected in bottled fruit.

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THE CHRYSANTHEMUMCARBOXYLIC ACIDS. VII.*—Catalytic Hydrogenation of the Chrysanthemic Acids

By S. H. HARPER

The chrysanthemic acids are catalytically hydrogenated to the stereoisomeric dihydrochrysanthemic acids. These acids are characterized, and esterified with allylrethrolone and propylrethrolone to give a series of esters for insecticidal testing.

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A dihydrochrysanthemic acid (3-*iso*butyl-2 : 2-dimethyl*cyclo*propane-1-carboxylic acid) was first prepared by Staudinger & Ruzicka[1] by the catalytic hydrogenation of naturally derived (+)-*trans*-chrysanthemic acid (3-*iso*but-1'-enyl-2 : 2-dimethyl*cyclo*propane-1-carboxylic acid) over Paal–Skita palladium, and later by Yamamoto,[2] using platinum black. The ester of this acid with naturally derived pyrethrolone was found to be insecticidally inactive towards cockroaches.[3] Subsequently the same acid was obtained by Haller & LaForge[4] and by LaForge & Acree[5] by the catalytic hydrogenolysis of cinerin-I and pyrethrin-I over Adams platinum, palladium-on-calcium carbonate, palladium-on-charcoal and Raney nickel. Later LaForge & Barthel[6] esterified this acid with naturally derived cinerolone and pyrethrolone and reported that the esters were half as toxic to houseflies as cinerin-I and pyrethrin-I respectively. With the availability from our earlier synthetic work[7, 8] of all the stereoisomeric chrysanthemic acids we have examined their catalytic hydrogenation and have prepared a number of esters of the resulting stereoisomeric dihydrochrysanthemic acids with allylrethrolone. The insecticidal testing of these esters should provide additional information about the effect of reduction of the *iso*butenyl group and of the stereochemistry of the side chain on toxicity.

Although LaForge & Acree[5] found 5% palladium-on-calcium carbonate to be the most effective catalyst for the rapid hydrogenolysis of cinerin-I and pyrethrin-I to the optically active *trans*-dihydrochrysanthemic acid (the sign of rotation of this acid appears never to have been recorded), in our hands it proved unsuitable for the reduction of either (±)-*trans*- or (±)-*cis*-chrysanthemic acid. Negligible reduction of the latter occurred and that of the former was exceedingly slow. In one experiment in which reduction of (±)-*trans*-chrysanthemic acid was apparently complete, the 'dihydro'-acid was obtained as a viscous liquid. Subsequent comparison of the refractive indices of this product, its chloride and allylrethronyl ester with those of the true dihydro-acid and its derivatives described below showed, in fact, that reduction had been incomplete. In retrospect, it is likely that the differing experiences of the efficacy of palladium-on-calcium carbonate in the reduction of *trans*-chrysanthemic acid were due to the widely differing catalyst-to-substrate ratios employed; whereas LaForge & Acree[5] used a catalyst-to-acid ratio of between 20 : 1 and 7 : 1 we used the more usual and economical ratio of 1 : 10—an hundredfold change, to our disadvantage. It may be remarked that Haller & LaForge[4] provided no rigid proof that their product was fully reduced; no crystalline derivatives were described and the liquid acid was characterized only by a carbon and hydrogen analysis and an approximate equivalent. We found also that no reduction of either (±)-*trans*- or (±)-*cis*-chrysanthemic acid occurred over 5% palladium-on-barium sulphate when catalyst-to-substrate ratios of 1 : 20 and 1 : 10 were used, or over Raney nickel.

Reduction over Adams platinum catalyst proved satisfactory, with catalyst-to-substrate ratios of 1 : 40, 1 : 50 or 1 : 100. Reduction was slow, consistent with the trisubstituted character of the ethylenic bond. In this way reduction of (±)-*trans*-chrysanthemic acid gave, on distillation, (±)-*trans*-dihydrochrysanthemic acid as a viscous liquid which crystallized to a low-melting solid on cooling; (±)-*cis*-chrysanthemic acid gave the crystalline (±)-*cis*-dihydrochrysanthemic acid directly. Reduction of (+)-*trans*- and (+)-*cis*-chrysanthemic acids was accompanied by a change of sign of optical rotation, for the resultant liquid dihydrochrysanthemic acids were laevorotatory.

No appreciable differences in rates of hydrogenation between *cis*- and *trans*-isomers were observed. In no preparative hydrogenation was the uptake of hydrogen significantly greater than that for one ethylenic bond. In fact, a quantitative microhydrogenation of (±)-*cis*-chrysanthemic acid over Adams catalyst in glacial acetic acid gave 1·01 double bonds. It is clear that there is no tendency for the *cyclo*propane ring in the chrysanthemic acids to undergo hydrogenolysis such as has been observed by Kierstead *et al.*[9] for the closely related 2-vinyl-*cyclo*propane-1 : 1-dicarboxylic acid under similar conditions.

None of the dihydrochrysanthemic acids gave a colour or precipitate with Denigès reagent, as do the chrysanthemic acids. This behaviour is consistent with our earlier suggestion[10] that interaction with Denigès reagent starts by hydration of the *iso*butenyl group.

Each dihydrochrysanthemic acid was converted by thionyl chloride into its chloride and this was esterified with allylrethrolone. The resulting mixed diastereoisomeric allylrethronyl esters were isolated by distillation. In every case in which the chloride was isolated by distilla-

tion it was accompanied by a substantial, sometimes preponderating, amount of the corresponding anhydride. This tendency of the dihydrochrysanthemic acids to form anhydrides with thionyl chloride appeared more marked than that of the chrysanthemic acids. Like *cis*-chrysanthemoyl chloride, *cis*-dihydrochrysanthemoyl chloride was more susceptible to hydrolysis by traces of moisture than the *trans*-isomer.

Inoue *et al.*[11] have recently prepared the mixed chrysanthemates of methyl-, ethyl- and propyl-rethrolone (methythrin, ethythrin and propythrin respectively), and claimed that these are as toxic, or nearly as toxic, as allethrin to houseflies; the criterion of effectiveness was median knock-down time. In apparent contrast Gersdorff[12, 13] had found only a low toxicity to houseflies for the esters of butyl- and amyl-rethrolone with (+)-*trans*-chrysanthemic acid, as had Haller & Sullivan,[14] and as was reported by Crombie *et al.*[15] for the esters of butyl- and amyl-rethrolone with (±)-*trans*- and (±)-*cis*-chrysanthemic acids. To provide additional esters for testing that may resolve this apparent discrepancy we have prepared propylrethrolone by catalytic hydrogenation of allylrethrolone over 5% palladium-on-barium sulphate. Reduction was rapid, consonant with the monosubstituted character of the side-chain ethylenic bond. Esterification of propylrethrolone with (±)-*trans*-chrysanthemic acid and (±)-*trans*-dihydrochrysanthemic acid gave esters that would constitute the more toxic components of dihydro- and tetrahydro-allethrin respectively.

These esters and others described in the Experimental section are being tested against the housefly by Dr. E. A. Parkin and Mr. A. A. Green of the Pest Infestation Laboratory, and against other species of insect by Dr. M. Elliott and Mr. P. Needham of Rothamsted Experimental Station. The results and a discussion of their bearing on the relationship of structure to toxicity will be published elsewhere.

Experimental

All rotations were determined in analytical-grade chloroform in a 2-dm. tube. Microanalyses are by Drs. Weiler and Strauss, Oxford.

Catalytic hydrogenation of the chrysanthemic acids.—Hydrogenations were carried out on 0·5–5 g. of acid in ethyl acetate in low-pressure glass apparatus at room temperature until absorption ceased. The products were isolated in 90–95% yield by filtration and subsequent distillation.

Reduction of (±)-*trans*-chrysanthemic acid (10 parts) over 5% palladium-on-calcium carbonate (1 part) extended over several days. One reduction was taken to apparent completion and gave a product, b.p. 134–135°/10 mm., n_D^{20} 1·456 (Found: C, 70·9; H, 10·8. $C_{10}H_{18}O_2$ requires C, 70·55; H, 10·65%) (with D. Thompson).

Reduction of (±)-*trans*-chrysanthemic acid (40, 50 or 100 parts) over pre-reduced Adams platinum oxide (1 part) required 50–100 minutes for completion. Distillation then gave (±)-trans-*dihydrochrysanthemic acid*, f.p. 23·0°, b.p. 132°/10 mm., 139°/15 mm., n_D^{20} 1·4482 (Found: C, 70·95; H, 10·7. $C_{10}H_{18}O_2$ requires C, 70·55; H, 10·65%).

Reduction of (+)-*trans*-chrysanthemic acid (50 parts) [*ex* ' polymerized pyrethrins ', b.p. 144–145°/14 mm., n_D^{20} 1·4738, $[\alpha]_D^{20}$ + 22·5° (*c*, 4·924)] over Adams platinum oxide (1 part) required 90 minutes for completion. Distillation then gave (−)-*trans*-dihydrochrysanthemic acid, b.p. 136°/16 mm., n_D^{20} 1·4474, $[\alpha]_D^{20}$ − 26·75° (*c*, 4·150). As Campbell & Harper[8] found $[\alpha]_D$ + 26° for pure (+)-*trans*-chrysanthemic acid, it appears that pure (−)-*trans*-dihydrochrysanthemic acid will have $[\alpha]_D$ approx. − 31°.

Reduction of (±)-*cis*-chrysanthemic acid (40 parts) over pre-reduced Adams platinum oxide (1 part) required 100 minutes for completion. Distillation then gave (±)-cis-*dihydrochrysanthemic acid*, b.p. 122°/7 mm., 125°/10 mm, which solidified and on crystallization from ethyl acetate separated as irregular masses, m.p. 55° (Found: C, 70·55; H, 10·7. $C_{10}H_{18}O_2$ requires C, 70·55; H, 10·65%) (with D. Thompson and R. A. Thompson).

Reduction of (+)-*cis*-chrysanthemic acid (50 parts) over Adams platinum oxide (1 part) required 70 minutes for completion. Distillation then gave (−)-cis-*dihydrochrysanthemic acid*, b.p. 137°/14 mm., which did not solidify on keeping at − 5°, n_D^{20} 1·4509, $[\alpha]_D^{20}$ − 50·2° (*c*, 5·018), $[\alpha]_D^{18}$ − 50·7° (*c*, 5·334) (Found: C, 70·65; H, 10·75. $C_{10}H_{18}O_2$ requires C, 70·55; H, 10·65%).

Ethereal diazomethane was added to a solution of (±)-*trans*-dihydrochrysanthemic acid

(3·5 g.) in ether until the yellow colour persisted. The solution was washed successively with dilute hydrochloric acid, sodium carbonate and water. Distillation of the dried solution then gave *methyl* (±)-trans-*dihydrochrysanthemate* (3·0 g., 80%), b.p. 86°/13 mm., n_D^{20} 1·4405 (Found: C, 71·85; H, 10·5. $C_{11}H_{20}O_2$ requires C, 71·7; H, 10·9%) (with R. A. Thompson).

Similar treatment of (±)-*cis*-dihydrochrysanthemic acid (3·5 g.) gave *methyl* (±)-cis-*dihydrochrysanthemate* (3·2 g., 85%), b.p. 85°/13 mm., n_D^{20} 1·4382 (Found: C, 71·7; H, 10·9. $C_{11}H_{20}O_2$ requires C, 71·7; H, 10·9%) (with R. A. Thompson). Both esters have pleasant odours similar to those of the methyl chrysanthemates.

Dihydrochrysanthemoyl chlorides.—The acid chlorides were prepared by interaction of the acid with a slight excess of thionyl chloride in light petroleum (b.p. 40–60°) and isolated by distillation. The following were obtained from 0·5–2·5 g. of acid:

(±)-trans-*Dihydrochrysanthemoyl chloride* (52–75%), b.p. 84°/10 mm., 88°/12 mm., n_D^{20} 1·4568 (Found: C, 64·05; H, 9·2. $C_{10}H_{17}OCl$ requires C, 63·65; H, 9·1%). One preparation gave a higher-boiling fraction, presumably the *anhydride* (41%), b.p. 183–187°/12 mm., n_D^{20} 1·4578, for hydrolysis regenerated (±)-*trans*-dihydrochrysanthemic acid.

(±)-cis-*Dihydrochrysanthemoyl chloride* (25%), b.p. 96–99°/16 mm., n_D^{20} 1·4595 (Found: C, 64·15; H, 9·3. $C_{10}H_{17}OCl$ requires C, 63·65; H, 9·1%), together with the *anhydride* (57%), b.p. 190–192°/16 mm., n_D^{20} 1·4605.

The high carbon and hydrogen percentages for these two acid chlorides suggest that slight hydrolysis had occurred between preparation and analysis.

(±)-trans-*Dihydro-δ-methoxychrysanthemoyl chloride* (53%), b.p. 100–101°/10 mm., n_D^{20} 1·4612, from (±)-*trans*-dihydro-δ-methoxychrysanthemic acid[16] (with D. Thompson).

(±)-cis-*Dihydro-δ-methoxychrysanthemoyl chloride* (61%), b.p. 100°/10 mm., n_D^{20} 1·4690, from (±)-*cis*-dihydro-δ-methoxychrysanthemic acid[16] (with D. Thompson).

*cyclo*Propanecarbonyl chloride (62%), b.p. 119–122°, from *cyclo*propanecarboxylic acid prepared by the method given in *Organic Syntheses*[17] (with M. A. Kazi).

Other acid chlorides were used for esterification without isolation; the light petroleum was distilled off, benzene added and the excess of thionyl chloride removed by distillation of a little of the benzene.

Esters of allylrethrolone.—Allylrethrolone was esterified with the appropriate acid chloride in benzene containing pyridine and the ester isolated by the procedure of Crombie *et al.*;[18] it was, however, found preferable to use equimolecular proportions of keto-alcohol and acid chloride, to remove the pyridine by first extracting with dilute hydrochloric acid, and to distil rapidly in a real vacuum of 10^{-1}–10^{-2} mm. rather than slowly in an apparent vacuum of 10^{-3} mm.

Allylrethrolone (1·12 g.) and (±)-*trans*-dihydrochrysanthemoyl chloride (1·40 g.) gave (±)-*allylrethronyl* (±)-trans-*dihydrochrysanthemate* (1·73 g., 77%), b.p. 136°/0·09 mm., n_D^{20} 1·4832 (Found: C, 75·05, 74·5; H, 10·0, 9·6. $C_{19}H_{28}O_3$ requires C, 75·0; H, 9·3%).

Allylrethrolone (0·38 g.) and (±)-*cis*-dihydrochrysanthemoyl chloride (0·33 g.) gave (±)-*allylrethronyl* (±)-cis-*dihydrochrysanthemate* (0·42 g., 55%), b.p. 128–132°/0·06 mm., n_D^{20} 1·4847 (Found: C, 75·3, 73·9; H, 9·1, 9·4. $C_{19}H_{28}O_3$ requires C, 74·95; H, 9·25%).

Allylrethrolone (0·30 g.) and (±)-*trans*-dihydro-δ-methoxychrysanthemoyl chloride (0·32 g.) gave (±)-allylrethronyl (±)-*trans*-dihydro-δ-methoxychrysanthemate (0·16 g.), n_D^{20} 1·4910 (with D. Thompson).

Allylrethrolone (0·35 g.) and (±)-*cis*-dihydro-δ-methoxychrysanthemoyl chloride (0·40 g.) gave (±)-allylrethronyl (±)-*cis*-dihydro-δ-methoxychrysanthemate (0·39 g.), n_D^{20} 1·4960 (with D. Thompson).

Allylrethrolone (1·00 g.) and the chloride from (+)-*cis*-chrysanthemic acid (1·11 g.) gave (±)-*allylrethronyl* (+)-cis-*chrysanthemate* (1·33 g., 67%), b.p. 130°/0·08 mm., n_D^{20} 1·506 (Found: C, 75·9; H, 9·0. $C_{19}H_{26}O_3$ requires C, 75·5; H, 8·7%).

Allylrethrolone (1·10 g.) and the chloride from (−)-*cis*-chrysanthemic acid (1·09 g.) gave (±)-*allylrethronyl* (−)-cis-*chrysanthemate* (0·70 g., 34%), b.p. 117°/1 × 10^{-3} mm., n_D^{20} 1·505–1·508 (Found: C, 75·9; H, 8·8. $C_{19}H_{26}O_3$ requires C, 75·5; H, 8·7%) (with L. R. Cox).

Allylrethrolone (5·80 g.) and *cyclo*propanecarbonyl chloride (4·00 g.) gave (±)-*allylrethronyl* cyclo*propanecarboxylate* (5·50 g., 66%), b.p. 103–107°/0·09 mm., n_D^{20} 1·504–1·505 (Found: C, 71·2; H, 7·65. $C_{13}H_{16}O_3$ requires C, 70·9; H, 7·35%). Light absorption: $\lambda_{max.}$ 2270 Å

(ε 15,300) in ethanol (with M. A. Kazi). LaForge *et al.*[19] have tested this ester without reporting any details of its preparation or physical properties.

Allylrethrolone (1·20 g.) and the chloride from methyl hydrogen (±)-*trans*-caronate (1·00 g.)[16] gave (±)-*allylrethronyl methyl* (±)-trans-*caronate* (0·82 g., 46%), b.p. 110–150°/6 × 10^{-3} mm., n_D^{20} 1·505 (Found: C, 68·75; H, 7·75. $C_{17}H_{22}O_5$ requires C, 66·65; H, 7·24%) (with R. E. Stedman).

Catalytic hydrogenation of allylrethrolone.—Reduction of allylrethrolone (5·0 g.) in methanol over 5% palladium-on-barium sulphate (0·5 g.) was rapid, and absorption of hydrogen ceased at the equivalent of one ethylenic bond. Distillation gave propylrethrolone (4·7 g., 93%), b.p. 101°/0·07 mm., n_D^{20} 1·494. Light absorption: $\lambda_{max.}$ 2300 Å (ε 9100) with a Hilger Medium Quartz spectrograph, but $\lambda_{max.}$ 2320 Å (ε 10,300 rising to 11,600 in the course of 1–2 days) with a Unicam SP.500 spectrophotometer, both in ethanol. Katsuda *et al.*[20] record b.p. 108–110°/0·5 mm. for propylrethrolone prepared by cyclization of 3-hydroxynonane-2 : 5-dione.

Other esters of the chrysanthemic acids.—Propylrethrolone (3·08 g.) and the chloride from (±)-*trans*-chrysanthemic acid (3·36 g.) gave (±)-*propylrethronyl* (±)-trans-*chrysanthemate* (4·40 g., 72%), b.p. 140–143°/0·09 mm., n_D^{20} 1·4975 (Found: C, 76·15; H, 9·4. $C_{19}H_{28}O_3$ requires C, 75·0; H, 9·3%).

Propylrethrolone (1·30 g.) and (±)-*trans*-dihydrochrysanthemoyl chloride (1·60 g.) gave (±)-*propylrethronyl* (±)-trans-*dihydrochrysanthemate* (1·78 g., 69%), b.p. 132°/0·07 mm., n_D^{20} 1·4736 (Found: C, 74·1; H, 9·95. $C_{19}H_{30}O_3$ requires C, 74·45; H, 9·9%).

(±)-*cis*-Cinerolone (2·7 g.)[21] and the chloride from (±)-*cis*-chrysanthemic acid (2·5 g.) gave (±)-cis-*cineronyl* (±)-cis-*chrysanthemate* (3·6 g., 77%), b.p. 120–155°/4 × 10^{-3} mm., n_D^{20} 1·507–1·510 (Found: C, 75·35; H, 9·0. $C_{20}H_{28}O_3$ requires C, 75·9; H, 8·9%). A forerun slowly deposited crystals of (±)-*cis*-chrysanthemic acid (180 mg.), m.p. and mixed m.p. 114° (with R. E. Stedman).

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THE SEED FAT OF *OMPHALEA QUEENSLANDIAE*

By H. H. HATT and A. Z. SZUMER

The large seeds of *Omphalea queenslandiae*, a member of the Euphorbiaceae, contain 28% of an edible, semi-drying oil. Its component glycerides consist almost entirely of those of the four common fatty acids, which in one sample were present in the proportions: palmitic 12·7, stearic 8·1, oleic 47·0 and linoleic 31·7%. The composition of the oil is variable.

Omphalea is a genus of the family Euphorbiaceae and its members are found mostly in tropical Central and South America. According to Jumelle,[1] three species occur elsewhere, one in Madagascar, one in the Philippines and one in Australia. The fruits of this genus have large seeds (nuts) with thin seed coats and kernels rich in fat. The fats from two species, *O. diandra* Aubl. and *O. megacarpa* Hemel., are known in America as cavete oil, and have been reported to resemble castor oil in physiological action,[2] although their insolubility in alcohol shows they can have no close chemical resemblance. According to Howes,[3] the seed of *O. triandra*, the Jamaican cob nut, is edible after the embryo has been removed. The customary physical and chemical characteristics of the seed fats from some American species have been reported several times;[1, 4, 5] Callier[6] has briefly reported on the component fatty acids of one species. He found that the acids from the seed fat of *O. diandra* contained 20% of saturated acids, consisting almost entirely of palmitic acid, and that the remainder was about 48% of oleic acid and 32% of linoleic acid.

The Australian species, *O. queenslandiae* Bail., is found in tropical Queensland. It is a climbing plant extending more than 100 feet and its large globular fruits usually contain three thin-shelled seeds with large kernels. The kernel is stated to be edible.

The seeds we examined had an average weight of 20 g. The kernels formed 75% of the seed and contained 29% of water; by extraction they gave an oil which formed 52% of the dry weight of the kernel or 28% of the fresh seed. The low acetyl value of this oil (0·6) and its optical inactivity showed there was no close resemblance to castor oil. Yet, because of the size of the seed and its high fat content, a detailed examination has been made.

Two samples of seeds from different localities were examined and the fats from them showed appreciable differences in iodine value and in the content of diallylic glycerides. Their properties are summarized in Table I. The sample from Innisfail was small, and a detailed examination was made only of the Babinda sample.

Table I

Physical and chemical constants of the seed fat

Sample	1	2
Place of collection	Innisfail	Babinda
Date of collection	March, 1953	May, 1953
Fat content of whole seed, %	27·6	28·5
Refractive index, n_D^{25}	1·4690	1·4680
Optical rotation, $[\alpha]_D^{20}$	0·0°	0·0°
Saponification value	192·7	194·9
Iodine value (Wijs)	107·5	97·1
Conjugated dienoic glyceride, as linolein, %	< 0·1	< 0·1
Conjugated trienoic glyceride, as linolenin, %	0·0	0·0
Diallylic glyceride, as linolein by isomerization, %	41·3	32·4
Triallylic glyceride, as linolenin by isomerization, %	0·9	0·5

Composition of the fat

The fat contains little non-saponifiable matter (1%), yields 94·1% of fatty acids (Hehner value) and 9·1% of glycerol; it is therefore a true fat. Its probable chemical composition can be inferred from the preliminary chemical examination. The spectroscopic data, Table I, show that only small amounts of conjugated unsaturated acids and of linolenic acid can be present. Low Reichert–Meissl (0·23) and Polenske (0·33) values were obtained and require that very little of the lower fatty acids be present. The virtual absence of hydroxy-acids

has already been noted. Determination of saturated acids by Bertram's method gave a value of 19·4%. If now the 32·4% of diallylic glycerides be regarded as glycerides of linoleic acid, the observed iodine value, 97·1, agrees well with that calculated, 97·6, if the remaining 46·7% are glycerides of oleic acid.

Analysis of the methyl esters by fractional distillation has proved these suppositions correct and has also given the composition of the saturated acids. For analysis, the fatty acids were resolved into a fraction I, crystallizing from acetone at − 30°, and a soluble fraction II. The properties of the two fractions are given in Table II. Both were esterified quantitatively with

Table II

Crystallization of fatty acids from acetone

	Fraction I (crystallizing at − 30°)	Fraction II (soluble at − 30°)
Yield, %	35·1	64·9
Equiv. wt.	272·3	283·8
Saponification equiv. (esters)	285·8	296·8
Iodine value (esters)	38·4	127·2
Diallylic esters (%) (as linolein)	0·6	48·5
Triallylic esters (%) (as linolenin)	0·0	0·8

diazomethane and the esters were fractionally distilled in a spinning-band column by the method previously described.[7] A chaser was used. The distillation curves are given in Fig. 1. They show that the solid acids of fraction I consist almost entirely of the C_{16} and C_{18} families. The saponification and iodine values of the fractions from the distillation of fraction I were determined and used to calculate the composition of fraction I. The distillation curve for fraction II shows that it contains almost entirely esters of the C_{18} family and its composition was calculated from the saponification and iodine values, and the spectroscopic data for the whole fraction.

Methyl palmitate was identified in the first plateau fractions of distillation I and methyl stearate and methyl oleate in its second plateau fractions; linoleic acid was identified in the fatty acids by preparation of tetrabromostearic acid. Knowing the acids present, the percentage composition of the fatty acid mixture was calculated to be: palmitic acid 12·7, hexadecenoic acid 0·02, stearic acid 8·1, oleic acid 47·0, linoleic acid 31·7, linolenic acid 0·5.

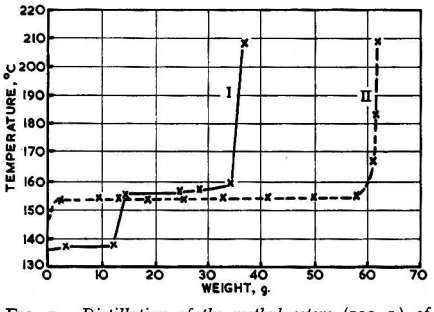

FIG. 1.—*Distillation of the methyl esters* (100 *g.*) *of fractions I and II from low-temperature crystallization of the fatty acids*

I From acids crystallizing at − 30°
II From acids soluble at − 30°

This composition for the fatty acids of *O. queenslandiae* is almost identical in oleic and linoleic acid contents with those reported by Callier for *O. diandra*. There is, however, a considerable difference in the composition of the saturated acids, for, unlike Callier, we have found a large proportion of stearic acid present. It appears that the agreement with his values for oleic and linoleic acid contents must be regarded as fortuitous because the composition of the fat in the Australian species is variable. Thus the Innisfail sample of fat almost certainly contains the same acids and its percentage composition calculated from iodine value and spectroscopic data must then be: saturated acids 18·1, oleic acid 39·7, linoleic acid 41·3, linolenic acid 0·9%.

Experimental

The methods employed were essentially those used in an earlier paper.[7] Some modifications in procedure and additional properties of the fat are given here.

The sample of seeds from Babinda (2392 g.) had an average weight of 20·8 g. It was

extracted 14 days after collection. The kernels were separated from the shells, chopped, partly dried at 50° under reduced pressure and extracted with light petroleum (free from aromatics, b.p. 60–80°) in an atmosphere of nitrogen. The solvent was removed on a water bath—the last traces under reduced pressure—and after a small amount of 'foots' had been removed on a filter aid a clear reddish-yellow liquid was obtained, possessing a faint nutty odour; yield 676 g. (28·5%). Properties of the fat measured and not given in Table I were: d_4^{25}, 0·9131; colour (Lovibond), 1·7 R, 23·0 Y, 6·0 N.T.; acid value, 1·3; peroxide value, 0·00.

The dry fat-free kernel contained N, 5·0% (protein, 32·5%) and yielded 9·9% of ash.

The fatty acids, obtained by hydrolysis in 94·1% yield, had a neutralization equivalent of 278·6; iodine value, 101·0; titre test, 29·1°; and a tetrabromide number of 30·4. The tetrabromide melted both alone (after crystallization from light petroleum), and also when mixed with an authentic specimen of 9 : 10 : 12 : 13-tetrabromostearic acid, at 114–114·5°. Low-temperature crystallization of the fatty acids was made from a 10% solution in acetone at − 30°; the crystalline fraction was recrystallized under the same conditions.

Methyl palmitate was isolated by repeated crystallization from methanol of the first plateau fractions of distillation I and melted at 30·0–30·7°. Methyl stearate isolated in like manner from the second plateau fractions had m.p. 38·5–39·3°. This same fraction when saponified and the acids oxidized with hydrogen peroxide and formic acid gave the low-melting form of 9 : 10-dihydroxystearic acid, m.p. 93–94°.

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Melbourne, Australia

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FUMIGATION OF AGRICULTURAL PRODUCTS. X.*—Sorption of Carbon Disulphide by Wheat and Flour †

By M. S. EL RAFIE

Previous accounts of the damage to wheat and wheat products caused by carbon disulphide fumigation are partly contradictory, and rarely describe accurate chemical measurements of sorption or the moisture content of the sorbent. The sorption is found to be critically dependent on moisture content, with a minimum sorption at about 14% of water. The sorption of carbon disulphide is even lower than that of methyl bromide, and seems to be mainly physical in origin. Most of the sorbed fumigant is recoverable, the remainder is held chiefly in the bran and endosperm. At high moisture contents, the seed-coat no longer inhibits sorption, which tends to the same limit as that exhibited by wholemeal flour.

Carbon disulphide, as a fumigant for grain, is widely used in tropical and sub-tropical countries, although it has been displaced by other materials in most temperate climates. Despite

* Part IX: *J. Sci. Fd Agric.*, 1954, **5**, 373
† Part of a Thesis approved for the degree of M.Sc., University of London

its widespread use few measurements of the sorption of carbon disulphide on grain or on grain products have been made, and those that have been reported are rendered of greatly reduced value because little attention has been paid to the moisture content of the fumigated products.[1] As Lubatti[2] has remarked, moisture content is as important a factor in determining sorption as, for example, the dose of fumigant applied.

Fumigation in airtight storage containers so prolongs the exposure of wheat to carbon disulphide vapour that airing seems to be necessary if retarded germination, or more serious damage, is to be avoided.[3] However, when the wheat is transferred to normally leaky storage conditions after fumigation, special airing seems unnecessary.[4] Even when the moisture content of the grain is known, discrepant results have been obtained with carbon disulphide when the wheat is tested for germination after airing. Endo[5] states that wheat is unaffected even when it contains 28·8% of water, but Annand[6] finds that germination is retarded if the wheat is fumigated when more than 12% of water is present. Evidently more information is needed about the quantity of fumigant sorbed by the stored products, and the nature of this sorption. The object of this paper is to provide such information.

Experimental

The investigation of the sorption of carbon disulphide was done in 1-l. glass chambers (Turtle chambers)[7] specially constructed for this kind of study. The carbon disulphide was introduced in a weighed, sealed ampoule, which was broken by vigorous shaking inside the Turtle chamber. Gas-concentration measurements were made by withdrawing samples of the air from the chamber, absorbing the fumigant in alcoholic caustic potash, and determining the carbon disulphide by the method of Higgins & Pollard.[8] The sampling technique has been reviewed in detail by Lubatti & Harrison.[9]

The method of recovery of sorbed fumigant from the wheat has been described by Lubatti,[10] whose ' wet aeration ' technique was used for most of these experiments, and who has described the precautions (temperature and dosage control etc.) that need to be observed during fumigation experiments of this kind.

Throughout this work clean English wheat, dried to a constant moisture content of 13%, was used as the sorbent unless otherwise stated. The temperature at which the experiments were done was controlled at 25°. All moisture contents were determined by the oven-drying method at 105°.

In some early experiments, the carbon disulphide recovered from whole grain, by ' wet aeration ' following a preliminary period of ' dry aeration ', was considerably less than the total amount sorbed. A substantial fraction of this residual fumigant was, however, found to be recoverable if the wheat were ground to flour in an airtight mill, and then subjected to the ' wet aeration ' process. The apparatus used for grinding wheat in an airtight mill has been described in full by Lubatti.[11]

Results

Preliminary experiments were made to estimate the rate of sorption of carbon disulphide by whole wheat, at two moisture contents. The lower moisture content (9%) is about the optimum for the prolonged storage of wheat. The higher value of 17·8% was chosen for comparison because Lubatti[2, 12] has shown how wetter wheat sorbs fumigants at rates more like those attained when flour is used instead of whole wheat.

Fig. 1 shows the results of these experiments. The total sorption is shown in the form of the Q_s/Q_f ratio introduced by Lubatti & Harrison.[9] This is a derived quantity intended to bring fumigations at different and usually falling concentrations to a comparable basis. In these experiments the initial concentration was 100 mg./litre. The total sorption of fumigant on the wheat is then expressed as a ratio, the divisor being the amount of fumigant remaining unsorbed. Since 100 g. of wheat is used in each experiment, the sorption ratio of 1 is attained, under these conditions, when one mg. of carbon disulphide has been sorbed per g. of wheat.

The results confirm Lubatti & Smith's findings[12] with methyl bromide fumigation of wheat, in that, at high moisture contents, the sorption of carbon disulphide is greatly increased for a given exposure period.

Influence of moisture content on sorption

A more detailed study was made of the sorption of carbon disulphide on wheat of different moisture contents, rather than of the rate of sorption.

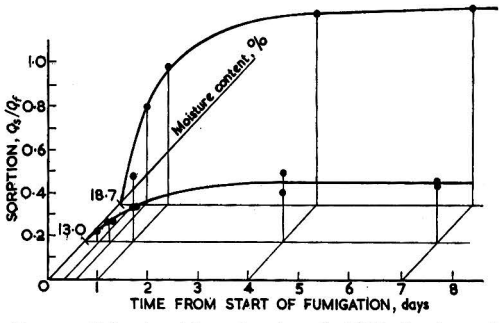

FIG. 1.—*Rate of sorption of carbon disulphide by dry and by damp grain*

Two sets of experiments were carried out, with wheat brought to the required moisture content either by drying it from an initial level of 18·7% or by wetting it from one of 7%. The results are given by Fig. 2. No hysteresis was detected between 'drying' and 'wetting' sorption curves represented as replicated points. Notable features are the low values obtained for the 'irrecoverable' fraction of the sorbed fumigant, and the minimum through which all the recovery curves pass, at moisture contents of about 14%.

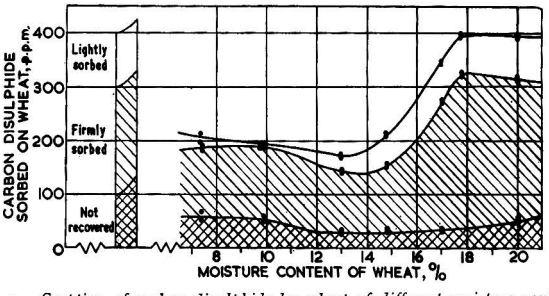

FIG. 2.—*Sorption of carbon disulphide by wheat of different moisture contents*

Sorption of carbon disulphide at different temperatures

Generally, sorption accompanied by chemical reactions increases with temperature, whereas predominantly physical sorption tends to decrease. The experiments whose results are illustrated by Fig. 3 were done on wheat of 13% moisture content. The results show that the recoverable fumigant is less strongly sorbed at high temperatures, and is presumably held by predominantly physical forces. The slight increase in the 'irrecoverable' fumigant may indicate some slight chemical reaction, but may reflect the deeper penetration of the fumigant at higher temperatures.

Investigation of sorption isotherms

Fig. 4 shows that the amounts of carbon disulphide sorbed on wheat are proportional to the partial pressure of the fumigant. Such curves are often called isotherms, although in fact equilibrium is not reached within the normal fumigation periods. These rectilinear sorption isotherms may assume the more familiar hyperbolic form when considerably wider ranges of concentration are investigated, although these higher concentrations are of less practical interest.

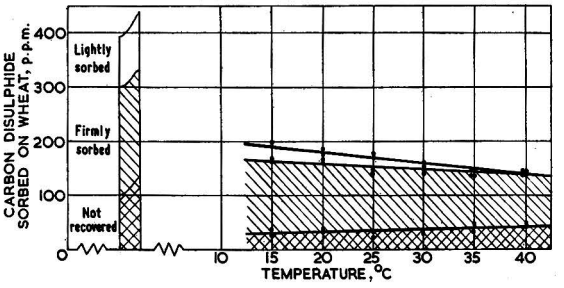

FIG. 3.—*Sorption of carbon disulphide by wheat at different temperatures*

Comparative sorption on wheat, germ, bran and on flour

In order to demonstrate the progressive failure of the seed-coat to control the rate of access of fumigants to the endosperm as the moisture content is increased, the results of comparable experiments with whole grain and wholemeal flour are given in Fig. 5. Lubatti & Smith[12] have discussed the interpretation of such experiments, with reference to the fumigation of wheat or onion seed with methyl bromide. As the water content is increased, the free surfaces available for sorption in the wholemeal flour appear to decrease, but for whole grains this effect is masked by the greatly increased permeability of the seed-coat, which with drier wheat renders the internal surfaces inaccessible. Fig. 6 represents experiments on wholemeal at different temperatures, similar to those reported for whole grains in Fig. 3. Fig. 6 also gives the corresponding results for patent (white) flour. The results are similar for all three materials. Sorption on the ground materials is higher than on whole grains, and that on wholemeal considerably greater than that on patent flour, from which the bran has been removed. The amount of unrecovered fumigant is much less dependent on either temperature or the nature of the flour.

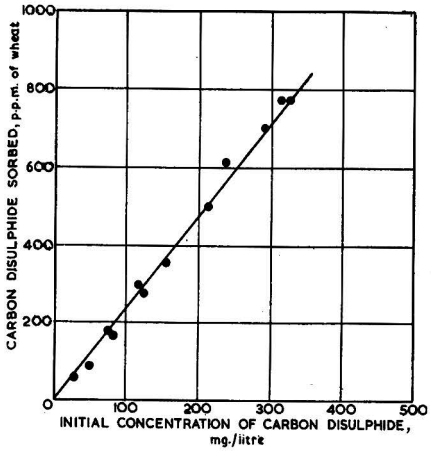

FIG. 4.—*Sorption isotherm of carbon disulphide on wheat at 25°*

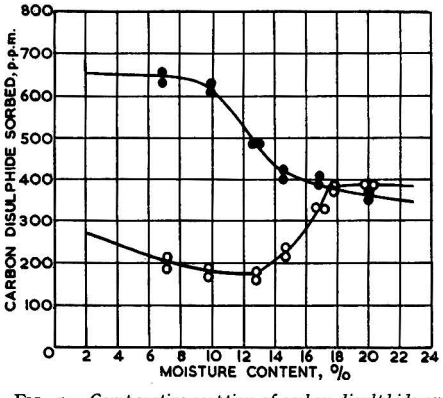

FIG. 5.—*Comparative sorption of carbon disulphide on flour and on grain*

Open circles: whole grains
Closed circles: wholemeal flour

Bran and germ from English wheat were brought to the same moisture content as the flour samples (13%). Fig. 7 gives the sorption of fumigant for whole wheat, wholemeal flour, patent flour, bran and germ under comparable conditions. The unrecovered fumigant appears to be held mainly in the endosperm, the chief constituent of the debranned, degermed, patent flour. Although the germ holds a relatively large amount of carbon disulphide, there is no evidence of any chemical reaction, all the sorbed fumigant being recoverable. Some caution is needed in comparing the quantities sorbed, first because

the proportion of the constituents is unequal in the whole grain or wholemeal flour, and secondly because the different constituents of the whole grain have different moisture contents when in equilibrium with a definite relative humidity. The total sorption on the whole grain at a given moisture content is not, in general, the weighted sum of the sorption on the constituent parts of the grain at that moisture content.

Discussion

The outstanding conclusions from this work arise when the results of the experiments reported here are compared with those reported on other fumigants.[9, 12] Sorption of methyl bromide is commonly regarded as low in comparison with that of ethylene oxide or of hydrogen cyanide. Of the amount of methyl bromide sorbed on wheat, a substantial proportion is not recoverable. Sorption of carbon disulphide is appreciably lower than that of methyl bromide. Moreover, an even smaller proportion of the sorbed carbon disulphide is not recoverable. A useful basis of comparison is the Q_s/Q_f ratio, which is 8·8, 2·4, 0·3 and 0·2 for hydrogen cyanide, ethylene oxide, methyl bromide and carbon disulphide respectively.

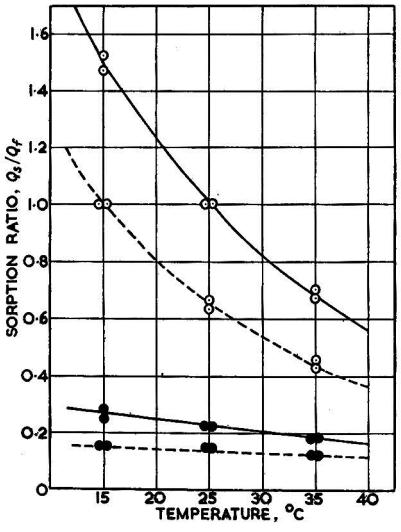

FIG. 6.—*Sorption of carbon disulphide on wholemeal and on patent flour, at different temperatures*

—⊙———⊙— Recoverable fumigant, wholemeal flour
—⊙- - - -⊙— Recoverable fumigant, patent flour
—●———●— Irrecoverable fumigant, wholemeal flour
—●- - - -●— Irrecoverable fumigant, patent flour

Elaborate arrangements for airing grain fumigated with carbon disulphide seem to be unnecessary. The ordinary processes of transport and milling are likely to remove about 70% of the recoverable fumigant. Increasing the period of fumigation beyond the customary periods of 24–48 hours produces but little increase in sorption, so that fumigation for a longer period, at low concentration, seems to be an efficient means of attaining a given concentration–time product. This conclusion is reinforced by the linear character of the sorption isotherms.

The importance of a low moisture content of wheat during fumigations scarcely needs emphasis. Increasing the moisture content of wheat from 13 to 18% more than doubles the total sorption of carbon disulphide and doubles the firmly retained fumigant. However, if wet wheat must be treated, some compensation may be derived from the fact that the open structure of damp grains readily allows desorption of carbon disulphide. Most of the small sorption is physical, and reversible on airing. The successful use of carbon disulphide without tainting the fumigated wheat is probably attributable to this. Since the sorption is mainly physical, it is little affected by the temperature at which fumigation is carried out. With most fumigants, sorption and its attendant risks of taint or loss of viability of seed increase sharply with temperature. The properties of carbon disulphide as a fumigant seem likely to show to greatest advantage under the

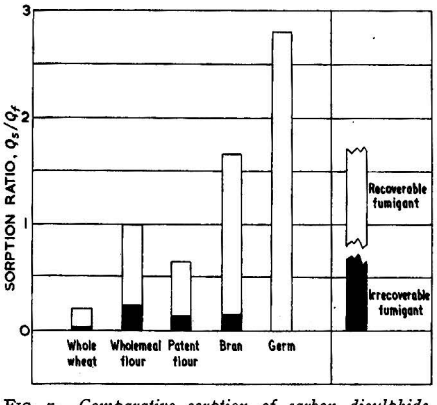

FIG. 7.—*Comparative sorption of carbon disulphide on wheat and on wheat products*

tropical conditions in which it has most firmly retained its place in the face of newer alternatives.

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THE USE OF ANTIBIOTICS IN THE FOOD OF FATTENING PIGS*

By K. L. ROBINSON, W. E. COEY and G. S. BURNETT

In a series of trials, weanling pigs were fed all-vegetable diets and diets containing fish meal, with and without procaine penicillin, under both *ad libitum* feeding and restricted feeding systems. The responses to the various treatments, as indicated by live-weight gains, suggest that factors that tend to promote keenness of appetite, such as antibiotics or fish meal, are favoured by *ad libitum* feeding, but do not exert their full effect with restricted feeding. These trials, and further trials with older pigs, confirm that responses to procaine penicillin are small after the 100-lb. live-weight stage, whether the supplement is first introduced then or has been fed continuously from an earlier period. The addition of a cyanocobalamin (vitamin B_{12}) supplement was found to enhance the growth-promoting effect of procaine penicillin on weanling pigs. Discontinuing the feeding of an antibiotic, after an initial response had been obtained, resulted in some degree of retardation.

The results for carcass conformation indicate no significant differences due to antibiotic treatments, and afford no evidence that such treatments result in commercially inferior carcasses.

Introduction

The main objective of this paper is to outline a series of trials in Northern Ireland when pigs were given dietary antibiotics, and to consider the results in relation to commercial pig fattening. When these trials were begun, in the middle of 1951, a number of brief communications on the subject already published in the U.S.A. had indicated that dietary antibiotics could produce considerable weight increases in weanling pigs. In a number of instances, however, the control pigs had made poor gains, so that even the accelerated gains of the treated pigs were little better than the average by U.K. standards. At about this time, however, a more detailed paper, from the Iowa Agricultural Experiment Station,[1] reported that with

* Read before the Agriculture Group on 16 February, 1954; for a report of the Discussion at the meeting see *Chem. & Ind.*, 1954, p. 484.

pigs gaining at the satisfactory rate of 1·25 lb./day over the range of 35–100 lb. the use of procaine penicillin gave an improvement of about 14%. It was thus difficult to resist the conclusion that the increases of up to 100% obtained at some of the other centres were perhaps brought about by antibiotics acting therapeutically on sub-normal animals.

A study of the growth curves in the Iowa experiment[1] indicated that the response to the antibiotic almost ceased after the 100-lb. stage had been reached, so that the over-all improvement at slaughter thus became reduced to about 8%. It followed that if responses of this type were representative of those attainable on reasonably good pigs, then the effects of antibiotic feeding could never be very great, judged by differences in final live-weight gains; an essential prerequisite of antibiotic feeding thus appeared to be that commercial supplementation should be inexpensive. Consequently we decided to base our own experiments on a low level of antibiotic, and to test the possibilities of removing the supplement from the diet after it had ceased to increase the rate of live-weight gain.

In assessing the applicability of the U.S.A. results to British practice some consideration had to be given to possible differences in methods of feeding and management. Practically all the U.S.A. results had been obtained with pigs fed *ad lib.* from automatic dry-meal feeders and with experimental designs based on group feeding; hence these results could be justifiably translated directly to U.S.A. commercial practice. Further, if, as had been reported,[2] the effect of antibiotics was mainly due to the enhanced appetite of the animals, the actual method of feeding might be a decisive factor. Thus it seemed to us important that the methods of feeding in our own experiments should be similar to those obtaining commercially in the U.K.

Experimental results

Individual-feeding trials

In our initial experiments[3] procaine penicillin (2 mg./1 lb. of meal) was added to an all-vegetable diet, based on groundnut meal, or to an animal-protein diet, based on white fish meal (Table II*a*). The food was trough fed as a wet mash and the scale of feeding employed, which was related to the live-weight gains, represented a 15% increase on the P.E.C.C. (Pig Experiments Co-ordination Committee) scale.[4] The results for the all-vegetable diet showed a 23% improvement in the live-weight of the treated pigs, over controls averaging 0·94 lb. daily throughout the 40–100-lb. weight range; with the fish-meal diet the improvement was 16% on controls averaging 1·1 lb. In both groups little further response occurred after the animals had reached 100 lb. live weight. It was observed that all the penicillin-treated pigs and the fish-meal controls had keener appetites than the all-vegetable controls, and the im-

Table I

Mean values for gain in weight, and food utilization on different scales of restricted feeding (from Robinson, Coey & Burnett[5])

	Diet						Coefficient of variation	Differences necessary to show significance at		
	1 All-vegetable	2 All-vegetable + penicillin	3 Fish meal	4 Fish meal + penicillin	5 Fish meal	6 Fish meal + penicillin		5%	1%	0·1%
			P.E.C.C. scale		P.E.C.C. scale +15%					
Live-weight increase (L.W.I.), lb.										
1st nine weeks	46·0	57·6	54·8	63·2	64·2	73·2	10·591	8·4	11·4	15·4
Meal/1 lb. of L.W.I., lb.										
1st nine weeks	3·55	3·09	3·07	2·86	2·91	2·82	5·845	0·24	0·32	0·43
Meal consumption, lb.										
1st nine weeks	162·8	177·0	167·9	180·5	185·2	206·0	10·916	25·9	35·3	47·8
Daily L.W.I., lb.										
1st nine weeks	0·73	0·92	0·87	1·00	1·02	1·16				
Daily L.W.I., lb.										
40–200	1·05	1·14	1·15	1·14	1·25	1·26	4·625	0·06	0·09	0·12

pression was gained that the scale of feeding employed had, in the early stages, resulted in some degree of under-feeding, and over-feeding, respectively.

The effect of scale of feeding on the response to procaine penicillin was next examined.[5] The diets and level of antibiotic were as used previously, but two scales of feeding were employed. The all-vegetable and fish-meal diets were compared on the P.E.C.C. scale, and the fish-meal diet was also fed on a scale 15% greater. As a measure of appetite the times taken to consume the rations at three successive evening feeds were recorded when the animals were between 60 lb. and 70 lb. in weight.[6] The effect of scale of feeding on live-weight gains is indicated in Table I, and the effect of diet and antibiotic on appetite in Table II. From these results it is evident that procaine penicillin increased the rapidity of eating of both diets and that the lower scale of feeding appreciably reduced the live-weight gains attainable.

Table II

Time taken to eat rations: mean of 3 feeds, diets and feeding as for Table I (from Robinson, Coey & Burnett[6])

Live weight of pigs on 29/1/52, lb.	61·8	67·4	64·8	70·8	66·4	72·4	12·653
Food consumed/pig/feed, lb.	1·34	1·50	1·46	1·60	1·70	1·76	10·822
Time taken to eat ration, min.	25·7	19·6	18·5	14·9	27·9	24·6	23·258*
Time taken/1 lb. of food, min.	19·6	13·9	12·7	10·0	16·6	14·9	23·213
Time taken to eat ration, min. (corrected for live-weight differences)	24·5	19·7	17·9	15·6	27·7	25·7	21·904

* Diff. necessary to show significance between any 2 treatment means, at 5% level = 6·7 min.
" " " " " " " 2 means, each of 3 treatments, at 5% level = 3·87 "
" " " " " " " " " " " " " 1% " = 5·28 "
" " " " " " " " " " " " " 0·1% " = 7·14 "

The fish-meal diet used in these trials can be considered as representative of 'sow and weaner' meals. It would be unusual, and perhaps even undesirable, for fattening pigs to be given this type of diet until slaughter, and in practice an all-vegetable, low-protein meal is generally introduced during the later stages of fattening; during the change-over some degree of set-back may occur. Thus in examining the commercial possibilities of antibiotics this alteration in diet must be given attention. In the trial summarized in Table I all the animals reverted to the all-vegetable control diet (without antibiotic) on the lower scale of feeding from 120 lb. live weight. It was observed that the pigs deprived of both fish meal and antibiotic suffered an impairment of appetite for a few weeks after the change, but the other groups showed no obvious effects. The final live-weight gains indicate that the earlier advantage in weight of the pigs receiving antibiotics was retained only on the all-vegetable diet.

Table II*a*

Composition of diets in Tables I and II

Foods, %	All-vegetable diet	Fish-meal diet
Groundnut meal	20	5
White fish meal	—	10
Barley meal	37	37
Bran	20	25
Maize meal	20	20
Grass meal	3	3
Minerals	2 lb./cwt.	—

These trials suggest that the conventional allowances of food, in the experimental method of feeding according to live weight, unduly limit the potential growth of pigs receiving fish-meal diets or diets containing an antibiotic. The system, moreover, has no particular relevance to commercial practice, in which pigs at the early stages of fattening are generally fed either *ad lib.* with dry meal or to appetite twice or three times daily with wet mash. It was decided, therefore, that in the succeeding individual-feeding trials (Tables III and IV) the animals would be fed to appetite three times daily.

In the trial summarized in Table III the standard all-vegetable diet was included, together with two fish-meal diets, one containing 7% and the other 12% of fish meal; procaine penicillin was again fed at 2 mg./1 lb. of meal. The weight gains of the control group were themselves

satisfactory but none the less responses of about 15 and 8% were obtained on the all-vegetable and fish-meal diets, respectively, up to the 100-lb. stage. The effect of the higher level of fish meal was not well defined. Here again there was no further antibiotic response on the fish-meal diet after the 100-lb. stage, but improvement continued on the all-vegetable diet. When the pigs reached 150 lb. live weight they were all given a finishing diet containing 5% of groundnut meal, 37% of barley meal, 30% of bran, 25% of maize meal and 3% of grass meal, together with a mineral supplement. No change was made in the antibiotic, which continued to be fed to the appropriate groups throughout. Although the results for this last stage of fattening are not clear-cut, they convey the impression that pigs receiving antibiotics may be particularly sensitive to marked dietary changes.

Table III

Mean values for gain in weight, and food utilization: penicillin throughout, all-vegetable diet from 150 lb. wt.

		Diet						Coefficient of variation	Difference necessary to be significant at		
		1 All-vegetable	2 All-vegetable + penicillin	3 Fish meal, 7%	4 Fish meal, 7%, + penicillin	5 Fish meal, 12%	6 Fish meal, 12%, + penicillin		5%	1%	0·1%
Daily L.W.I., lb.	45–100	0·99	1·14	1·22	1·31	1·27	1·38	8·3	0·13	0·18	0·24
,, ,, ,,	100–150	1·47	1·65	1·74	1·70	1·76	1·73	10·6			
,, ,, ,,	150–200	1·70	1·73	1·82	1·51	1·54	1·52	11·4			
,, ,, ,,	45–200	1·28	1·37	1·52	1·47	1·49	1·51	6·2	0·12	0·16	0·22
Meal/lb. of L.W.I., lb.	45–100	3·16	2·95	2·91	2·79	2·69	2·61	6·9	0·26	0·35	0·48
Meal/lb. of L.W.I., lb.	45–200	3·88	3·57	3·43	3·63	3·51	3·48	5·1	0·24	0·33	0·44

The results of a further trial carried out with feeding to appetite are given in Table IV. The basal fish-meal diet was modified in the various treatments by the addition of procaine penicillin, liver meal, liver meal + procaine penicillin, vitamin B_{12} (22 μg./1 lb. of meal) procaine penicillin or Aurofac 2A [a chlortetracycline (aureomycin) supplement containing 3·6 g. of

Table IV

Mean values for gain in weight, and food utilization: penicillin removed at 120 lb. wt.

		Diet						Coefficient of variation	Difference necessary to be significant at		
		1 Fish meal, 10%	2 Fish meal, 10%, + penicillin	3 Fish meal, 5%, liver meal, 5%	4 Fish meal, 5%, liver meal, 5%, + penicillin	5 Fish meal, 10%, + B_{12}, + penicillin	6 Fish meal, 10%, + Aurofac		5%	1%	0·1%
Daily L.W.I., lb.	40–120	1·23	1·27	1·14	1·33	1·34	1·63	5·762	0·10	0·14	0·18
,, ,, ,,	120–200	1·72	1·76	1·90	1·70	1·81	2·16	9·241	0·22	0·31	0·38
,, ,, ,,	40–200	1·43	1·47	1·42	1·49	1·53	1·86	6·737	0·14	0·19	0·25
Meal/lb. of L.W.I., lb.	40–120	2·82	2·74	3·00	2·72	2·67	2·42	5·430	0·20	0·27	0·36
Meal/lb. of L.W.I., lb.	40–200	3·36	3·27	3·28	3·37	3·21	3·01	6·246	—	—	—
Meal consumption in 1st 4 weeks, lb.		69·6	68·3	68·1	71·2	69·7	85·8				
Weekly L.W.I., wk. before	120 lb.	11·8	12·0	11·4	12·2	13·2					
Weekly L.W.I., 1st wk. after	,,	10·2	12·2	13·2	9·8	8·8					
Weekly L.W.I., 2nd wk. after	,,	11·4	10·3	13·2	12·8	13·6					

Table IV*a*

Carcass measurement results for treatments 1 *and* 6, *Table IV*

	1 Control	6 Aurofac	Coefficient of variation	Significance
Cold carcass wt., lb.	138	146	3·204	None
Killing out, %	72·5	72·6	2·134	,,
Length of carcass, mm.	747	773	2·450	,,
,, ,, hind leg, mm.	571	580	2·022	,,
Depth, mm.	322	327	2·451	,,
Shoulder fat, mm.	47·0	48·2	8·701	,,
Loin fat, mm.	17·6	18·0	22·501	,,
Rump fat 1, mm.	29·4	32·2	17·086	,,
,, ,, 2, mm.	27·2	29·4	20·908	,,
,, ,, 3, mm.	34·0	35·4	18·128	,,
Belly thickness, fore, mm.	35·4	35·8	17·749	,,
,, ,, mid, mm.	31·0	33·8	8·201	,,
,, ,, hind, mm.	35·0	37·2	7·000	,,

chlortetracycline hydrochloride per lb. together with an appreciable but unspecified amount of cyanocobalamin] (8 lb./1 ton of meal). For reasons to be considered below, the Aurofac supplement was continued throughout, but when penicillin was given it was withdrawn at 120 lb. live weight. Up to the 120-lb. stage the daily live-weight gains of the control group averaged 1·23 lb., a figure that penicillin was unable to raise. Somewhat surprisingly the liver-meal group (5% of fish meal + 5% of liver meal in place of 10% of fish meal) did less well than the controls, but this diet produced a very significant response when combined with penicillin; penicillin + vitamin B_{12} also gave a significant response over the controls. The removal of penicillin at 120 lb. live weight did not appear to cause much recession, although the weekly weight-gain figures suggest a temporary set-back in those groups that had responded to the antibiotic. The figures for food consumption during the initial four weeks of the experiment suggest that the differences in live weight obtained were largely due to a greater efficiency in food utilization and not to increased food-intake.

The behaviour of the Aurofac group was noteworthy, and in our experience exceptional. From the outset these pigs had very keen appetites and made very rapid growth up to the 120-lb.-weight stage. When Aurofac was removed from the diet of the first pig, its food consumption dropped rapidly and the animal went into a rapid decline. The antibiotic was restored to the food and effected a speedy recovery, so that the final weight gains were excellent. The second pig behaved similarly but less dramatically; consequently it was decided to allow the remainder to continue unchecked. The growth curves for the first two pigs are shown in Fig. 1. Two subsequent individual-feeding trials have been undertaken with use of Aurofac 2A (5½ lb./1 ton of meal) throughout and with no dietary changes. In one the standard 10% fish-meal diet was used with restricted feeding (P.E.C.C. scale + 10%) and in the other an all-vegetable diet was fed to appetite three times daily. The responses obtained were of the same order as those in the earlier trials with procaine penicillin, and the growth curves (Fig. 2) indicate that improvement again ceased at about the 100-lb. stage. The responses of the fish-meal group cannot be directly compared with those in the earlier Aurofac trial, since feeding was not to appetite and the behaviour of the animals left little room to doubt that they were being appreciably underfed. The all-vegetable groups did not suffer this restriction, and the poorer results, which are very similar to the comparable ones in the penicillin trials, are presumably in part due to the shortcomings of the basal diet.

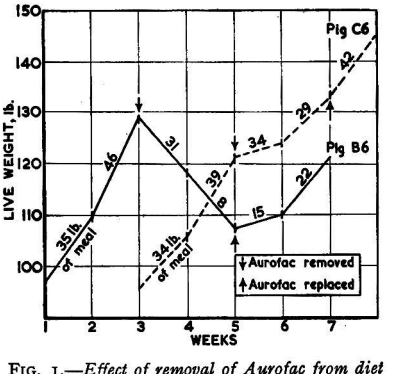

FIG. 1.—*Effect of removal of Aurofac from diet on live weight*

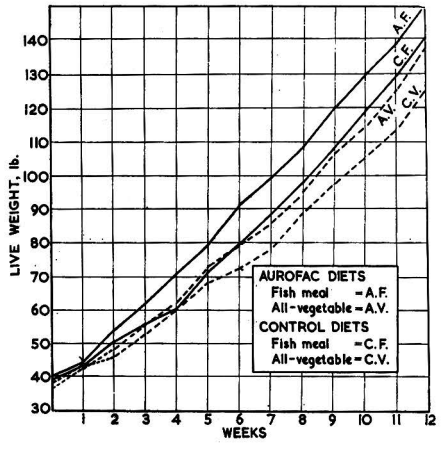

FIG. 2.—*Effect of continuous use of Aurofac on live weight*

Group-feeding trials

A number of our group-feeding trials have already been reported,[6] but are briefly summarized now, with some additional results (Table V). In these trials there were about ten pigs in each pen, feeding *ad lib.* from dry meal feeders, with water always available at automatic drinking bowls. These trials were designed to test earlier results under more exacting commercial conditions and at contrasting times of the year (January and August). They were also intended to examine further the question of diminishing response to antibiotics after the 100-lb. stage. No evidence of increased consumption of food due to antibiotics was obtained in any live-weight range with fish-meal diets, and the greater weight gains up to the 100-lb. stage were apparently due to increased efficiency of food utilization. On the all-vegetable diets it appeared that antibiotics encouraged an increased food-intake with *ad lib.* feeding (Table V). After reaching 100 lb. weight some of the groups on the fish-meal diet were deprived

Table V

Live-weight gains and food consumption: group feeding

	10% Fish-meal diet				All-vegetable diets						
	Con-trol	Peni-cillin	Con-trol	Peni-cillin	Con-trol	Terra-mycin, 5 mg./lb.	Con-trol	Peni-cillin	Con-trol	Penicillin/lb. 2 mg.	5 mg.
Live weight at start, lb.	30	30	105	105	45	45	75	75	100	100	100
,, ,, ,, finish, lb.	100	100	210	210	150	150	220	220	220	220	220
Daily L.W.I., lb.	1·03	1·20*	1·68	1·75†	1·03	1·20	1·39	1·48	1·48	1·55	1·60
Meal/1-lb. gain, lb.	3·38	3·13	4·21	3·87	4·12	3·77	5·0	4·9	5·4	5·1	5·4
Meal eaten 1st 4 wk., lb.	67	72	248	244	—	—	140	174	154	202	199
Response	17% (mean of 4 trials)		4% (mean of 2 trials)		16% (mean of 2 trials)		6% (1 trial)		5% (1 trial)	8%	

* Significance at 0·1%
† Significance none

of penicillin and given a fattening diet containing 2½% of fish meal and 7½% of groundnut meal. The subsequent weight gains (Table VI) again indicate some degree of set-back from the dual change. With both the fish-meal and the all-vegetable diets, groups were included

Table VI

Penicillin stopped at 100 lb. live weight: group feeding

	Daily L.W.I., 100–210 lb.	
	No penicillin throughout	Penicillin until 100 lb. live weight
Exp. 1	1·66	1·59
Exp. 2	1·53	1·34
Exp. 3	1·52	1·59*
Exp. 4	1·47	1·33

* Penicillin treatment continued until slaughter

in which penicillin was introduced at 100 lb. live weight and continued until slaughter at 200 lb. The responses to this late introduction of antibiotic were small (Table V).

Carcass conformation

In the four individual-feeding trials already mentioned the animals were slaughtered when about 200 lb. in weight and carcass measurements were obtained according to the method of McMeekan.[7] Some comparisons of controls with penicillin-fed animals taken from these results are given in Table VII, and are based on 37 litter-mate pairs on fish-meal diets and

Table VII

Carcass measurements and live-weight gains

	Animal-protein diets				All-vegetable diets	
	Control	Penicillin	Coefficient of variation	Significance	Control	Penicillin
Daily L.W.I., lb., start–120 lb.	1·17	1·26	5·3	0·1%	0·98	1·11
,, ,, ,, start–slaughter	1·38	1·40	4·5	None	1·18	1·30
Meal/1 lb. of L.W.I., start–slaughter	3·40	3·43	1·8	None	3·70	3·51
Carcass weight, lb.	143·4	145·8	4·0	None	140·6	145·1
Killing out, %	73·88	73·75	1·7	None	73·71	73·87
Length, mm.	761·9	769·5	9·5	None	759·5	767·6
Backfat at shoulder, mm.	49·54	50·68	8·4	None	48·40	50·40

15 on all-vegetable diets. Any differences are only small, and are evidently due in the main to the slightly heavier carcasses of the penicillin-treated animals. Thus it would appear that in the absence of any marked improvement in over-all rate of live-weight gain, after an early response, penicillin had little, if any, effect on carcass conformation. The carcass measurement results from the pigs on the Aurofac trial summarized in Table IV have been presented in more detail (Table IV*a*), because of their special interest as resulting from response to an antibiotic of about 30% over-all weight gain. It is apparent that the considerable disparity in rate of growth has had remarkably little effect on carcass conformation, and here again such differences as do exist can be accounted for by the rather heavier carcasses of the antibiotic-fed pigs and would not adversely affect commercial grading.

Effect of procaine penicillin on the goitrogenic action of methyl thiouracil

Pigs are very sensitive to the thiouracils and we have already shown that substantial economies in food consumption can be secured by the use of thiouracil in the diet when restricted feeding is practised.[8] In some recent trials, on the *ad lib.* dry meal system, a saving in meal of over 40 lb. per pig has resulted from feeding methyl thiouracil at a level of 0·025%. Further, we have obtained evidence that methyl thiouracil has a vitamin-E-like effect in that it reduces the discoloration and peroxidation of pig backfat induced by diets high in cod-liver oil. The commercial potentialities of methyl thiouracil are, however, limited by the fact that continuous administration ultimately causes a sudden and pronounced drop in food consumption, which occurs the sooner the younger the pigs; thus whereas 140-lb. pigs can usually be safely brought to a slaughter weight of 200 lb., pigs of 100 lb. weight show a decline in appetite after three or four weeks. Libby & Meites[9] have reported that penicillin prevents the appetite drop in chicks without altering the other effects induced by thiouracil; this has led us to similar investigations with pigs. The growth curves shown in Fig. 3 are representative of the results obtained and indicate that, under our conditions, procaine

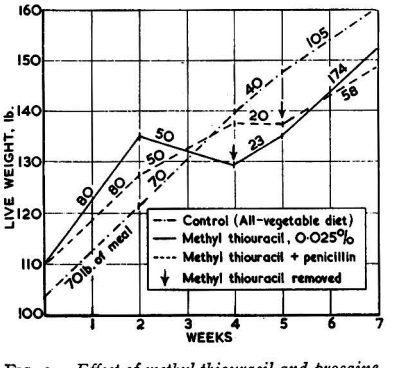

FIG. 3.—*Effect of methyl thiouracil and procaine penicillin on live weight*

penicillin at 2 mg./1 lb. of meal was not effective in counteracting the effect of methyl thiouracil on the appetites of fairly young pigs.

Discussion

It is evident that experimental scales of feeding based on live weight, such as the P.E.C.C. scale, are unfavourable to fish-meal diets and diets containing antibiotics, when comparisons are being made with all-vegetable diets and unsupplemented diets. The much more impressive results of the fish-meal controls in the 'to appetite' individual-feeding trials bear this out and at the same time tend to suggest that the pigs fed on an all-vegetable diet with unrestricted feeding would usually eat amounts of food similar to those permitted by the P.E.C.C. scale.

The inclusion of an antibiotic in a diet promotes a keenness for food and greater rapidity of eating. With the wet meal system it is customary to gauge food requirements by the amount the pigs will eat in a limited time, and it thus seems likely that the use of antibiotics will help pigs to secure a more generous ration for themselves when kept under this system.

The results of the unrestricted feeding trials with high-quality (fish-meal) diets indicate that when restrictions in food consumption, due either to a time factor or to a scale of feeding, are removed, procaine penicillin no longer promotes greater food intake, but an appreciable improvement in live-weight increase can still be obtained with weanling pigs owing to increased efficiency of food utilization. With poorer quality (all-vegetable) diets, however, the antibiotic still appears to exert an appetite effect even with unrestricted feeding.

In the trials in which an early weight-gain response was obtained by the inclusion of antibiotics, subsequent removal brought about some degree of retardation, possibly related in magnitude to the original response. An examination of these set-backs in the several trials suggests that a change-over from a fish-meal to an all-vegetable diet, such as occurs in normal farm practice, may induce as big a check as the removal of an antibiotic; since a dual change may enlarge the effect, the retention of the antibiotic throughout the entire feeding period seems desirable. When this is done the possibility still remains that the feeding of antibiotics renders pigs more susceptible to major dietary changes, and this point appears worth further investigation.

All the trials confirmed that responses to procaine penicillin after the 100-lb. live-weight stage were fairly small, whether the supplement was first introduced then or had been fed continuously from an earlier stage. It is possible, therefore, that the practice of buying-in store pigs of upwards of 70 lb. weight for fattening may render unattainable appreciable responses to feeding antibiotics.

In previous experiments[3] no responses were obtained from vitamin B_{12} on all-vegetable diets, so that the significant combination of B_{12}, and of liver meal, with procaine penicillin, obtained on fish-meal diets presumably already adequate in B_{12}, are difficult to interpret: these responses (Table IV) are not due to increased food intake.

The results for carcass conformation indicate no significant differences, and there is no evidence that the carcasses from the antibiotic-fed animals do in fact tend to be inferior in commercial grade. This was so even when wide differences in growth rates were obtained. These results are not surprising considered in relation to the classical investigations of growth and conformation in the pig. McMeekan & Hammond[10] showed that the most desirable commercial carcasses were produced when an initial high plane of nutrition was followed by a lower plane in the later stages of fattening. Winters, Sierk & Cummings[11] conclude that the most desirable results are likely to be obtained when pigs with a capacity for rapid growth are somewhat restricted in growth rate during the finishing period. The use of antibiotics would appear to be a step in this direction, since on the whole the supplemented animals grow more rapidly than the controls in the early stages and relatively less rapidly towards the end.

The results given in this paper include all the completed trials with antibiotics that we have carried out at the Agricultural Research Institute of Northern Ireland. In view of the inherent variability of the experimental animals, which were representative of commercial Large Whites, it is considered particularly worth emphasizing that an initial growth response to antibiotics was obtained in every trial, and it may be that the ability of antibiotics to secure a growth acceleration after weaning—probably the most difficult period in the husbandry of

the bacon pig—will justify their use commercially even when pronounced improvements in the over-all rate of live-weight gain are not obtained.

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STUDIES ON PROTEIN HYDROLYSIS. II.*—The Use of Sulphurous Acid for the Control of Humin Formation and Loss of Tryptophan during Acid Hydrolysis

By J. W. PEDERSEN and B. E. BAKER

The autoxidation of sulphurous acid has been investigated with the aim of finding suitable conditions under which the acid might be used, either alone or in the presence of strong mineral acids, for the hydrolysis of proteins.

Sulphurous acid alone was used to hydrolyse casein, lactalbumin, ovalbumin and fibrin to 34–37% of complete hydrolysis. Losses of tryptophan ranged from 0 to 3% of the total. Use of N-sulphuric acid with the sulphurous acid gave 65–70% hydrolysis with losses of tryptophan ranging from 2 to 10% of the total. Amounts of 10 g. of sulphur dioxide per 100 ml. of N-sulphuric acid and 15 g. of sulphur dioxide per 100 ml. of N-hydrochloric acid were required to give effective tryptophan preservation.

Preliminary rat-feeding tests indicated that a sulphurous acid–sulphuric acid casein-hydrolysate would support growth and that it was not grossly toxic.

Introduction

The literature dealing with the acid hydrolysis of proteins is very extensive, but only a relatively small fraction is concerned with practical methods of acid hydrolysis that avoid formation of humin and destruction of tryptophan.

Hlasiwetz & Habermann[1] found that colourless hydrolysates could be obtained by the use of a mixture of hydrochloric acid and stannous chloride. More recently Sullivan & Hess[2] suggested the use of a mixture of hydrochloric acid and titanous chloride. Sahyun[3] reported that a mixture of zinc dust or zinc chloride and hydrochloric acid was an effective hydrolytic agent and gave little humin formation.

Rigby[4] has described a method in which hydrogen is generated electrolytically in a

* Part I: *J. Sci. Fd Agric.*, 1952, **3**, 470

mixture of protein and sulphuric acid. The apparatus consists of two compartments, one of which contains a platinum anode and the other a lead cathode. Rigby secured a high degree of hydrolysis with the preservation of up to 70% of the original tryptophan of the protein. The hydrolysates prepared by this method were claimed to be colourless.

The use of sulphurous acid for the prevention of humin formation has received little attention. Monte & Gottfried[5] claimed, in a patent dealing with the extraction of corn protein at pH 1·0–1·2, that sodium bisulphite and sodium sulphite prevent the discoloration of the protein. Sulphurous acid has been used to extract the protein from solvent-extracted soya-bean meal[6, 7] and the extracted protein is claimed to have a superior pale colour.

The autoxidative properties of sulphurous acid limit its use as a hydrolytic agent. Little quantitative work on this reaction was reported until Jungfleisch & Brunel[8] and, later, Foerster & co-workers[9] published their studies on the autoxidation of sulphurous acid at elevated temperatures. Foerster & co-workers investigated the reaction at 120–125°, 150° and 180°. Their results show that, even at the lowest temperature, a heating period of 14 hours gives 37·5% conversion of the sulphurous acid into a mixture of elemental sulphur, sulphuric acid and thionic acids. Strong mineral acids, such as hydrochloric acid and sulphuric acid, were found to retard the autoxidation reaction.

The present paper deals with (*a*) the autoxidation of sulphurous acid at 100° and 110° and (*b*) the hydrolysis of proteins with sulphurous acid and with a mixture of sulphurous acid and strong mineral acids, with special emphasis on the avoidance of destruction of tryptophan during hydrolysis.

Methods

Hydrolysis with sulphurous acid in sealed tubes

A small cylinder of sulphur dioxide was cooled to — 16° and the desired volume was then run into a resistance-glass Carius tube. The protein was added to the tube, followed by the required weight of ice. The tube was sealed with a hand torch and was placed in a rocking device. The temperature of the tube could be maintained constant to within the range plus or minus 2°. The heating time was counted as from the moment the tubes were placed in the furnace and the current turned on, to the moment the current was turned off. Approximately three hours were required to raise the temperature of the furnace to 100° and approximately the same time was required for cooling to room temperature. A rocking motion of the tubes was maintained throughout the heating period.

Total nitrogen and amino-nitrogen analyses

Total nitrogen was determined by a semi-micro Kjeldahl method with a catalyst consisting of a mixture of cupric sulphate and mercuric sulphate.[10]

Amino-nitrogen was determined by the volumetric method of Van Slyke[11] after the sulphurous acid had been removed from the sample. For the removal of sulphurous acid the sample was evaporated, *in vacuo*, to half volume; sufficient distilled water was added to the sample to bring its volume to the original value and the evaporation was repeated.

Tryptophan

Tryptophan was determined by the modified glyoxylic acid method.[12] Before tryptophan was determined, the sulphurous acid hydrolysates were evaporated *in vacuo* as outlined above in connexion with the determination of amino-nitrogen.

Sulphate

In the study of the autoxidation of sulphurous acid, polythionates and sulphate were determined together gravimetrically after the solution had been evaporated to dryness to remove the sulphurous acid. The method used was as follows: After the autoxidation reaction, the mixture was filtered to remove elemental sulphur and filtrate was then evaporated to dryness on a steam bath. The residue was dissolved in a small quantity of water, 0·5 g. of sodium chlorate was added, the mixture was boiled for ten minutes and then evaporated to dryness.

The residue was dissolved in 400 ml. of water and the sulphate was determined in the usual way.[13] The results are reported in terms of the quantity of sulphur present.

Experimental

Autoxidation of sulphurous acid

Preliminary experiments showed that the determination of tryptophan by the glyoxylic acid method could not be performed successfully in the presence of greater than trace amounts of thionic acid. If the amount of tryptophan present in the sulphurous acid hydrolysates was to be determined by this method, it was necessary to hydrolyse under conditions that would not lead to autoxidation of the sulphurous acid.

In the following experiments the autoxidation reaction has been studied at 100° and 110°. The quantities of elemental sulphur and non-volatile sulphur-containing compounds formed in the reaction mixture have been used as a measure of the stability of the sulphur dioxide.

Autoxidation at 100° *and* 110°.—Liquid sulphur dioxide and ice were sealed in glass tubes and heated, with constant agitation, for various time intervals. The tubes were cooled and opened, the elemental sulphur was filtered off, washed with distilled water and dried to constant weight at 110°. The combined filtrate and wash water was evaporated to dryness, and the residue, which was a mixture of sulphuric acid and thionic acids, was treated with sodium chlorate. The total sulphur content of the residue was calculated from the weight of the sulphate formed. The results are shown in Table I.

The effects of casein on the autoxidation reaction.—A series of experiments were performed at 100° to find the effects of casein on the autoxidation reaction. A mixture of casein, water and sulphur dioxide was sealed in glass tubes and heated at 100° for various time intervals. At the end of the heating period the tubes were cooled and opened. The mixtures contained no elemental sulphur. The undissolved casein was filtered off, and the filtrate was evaporated to a small volume, *in vacuo*; this evaporation removed the sulphurous acid. The residue from the evaporation was treated with aqua regia to convert the non-volatile, sulphur-containing compounds into sulphate, from the weight of which the amount of sulphur in the residue was calculated. A correction was made for the sulphur in the residue that originated from the dissolved casein. The results are given in Table I. By comparing these results with those obtained in the previous experiments it may be seen that the casein had little or no effect on the formation of non-volatile, sulphur-containing compounds.

The effects of strong mineral acids on the autoxidation reaction.—Sulphur dioxide was heated in sealed tubes at 110° with dilute hydrochloric acid and with dilute sulphuric acid. In the experiments with hydrochloric acid the same procedure was followed as was outlined above. The results are shown in Table I. In the experiments with sulphuric acid the procedure was modified as follows. After the sulphur dioxide had been removed from the reaction mixture by evaporation *in vacuo*, the sulphate was precipitated with barium chloride. The amount of sulphate formed by the autoxidation reaction was calculated from the weight of this precipitate (P_1), after subtracting the amount of sulphate originally present in the reaction mixture. The combined filtrate and washings were evaporated to dryness and the residue was treated with sodium chlorate. The resulting sulphate was precipitated with barium chloride and the quantity of sulphur that was not removed by the first precipitation with barium chloride was calculated from the weight of this second precipitate (P_2). The results of these experiments are shown in Table I. Both the sulphate and the thionates (not precipitated by barium chloride) formed by the autoxidation reaction are expressed in terms of sulphur content. No elemental sulphur was formed in any of these experiments.

Hydrolysis of proteins with sulphurous acid

Mixtures of 5 g. of casein, 23 g. of sulphur dioxide and 50 g. of ice were sealed in glass tubes and heated at 100° with constant agitation. At the end of the heating periods the sulphur dioxide was removed from the hydrolysates by evaporation *in vacuo*. The hydrolysates were analysed for amino-nitrogen, total nitrogen and tryptophan. The percentage

Table I

The autoxidation of sulphurous acid

Reaction time, h.	Elemental sulphur, g.	Sulphur content of residue, g.
Reaction mixture: sulphur dioxide, 23 g.; water, 50 g. Temperature, 100°		
0	—	0·005
7	—	0·009
12	—	0·018
24	—	0·032
36	—	0·047
48	0·058	0·162
Temperature, 110°		
0	—	0·005
6	—	0·034
12	—	0·048
15	0·450	1·087
20	0·500	1·126
29	0·733	1·268
Reaction mixture: sulphur dioxide, 23 g.; water, 50 g.; casein, 5 g. Temperature, 100°		
0	—	0·060
7	—	0·013
12	—	0·020
24	—	0·032
36	—	0·048
Reaction mixture: sulphur dioxide, 23 g.; N-hydrochloric acid, 50 g. Temperature, 110°		
20	—	0·123
24	0·012	0·153
36	0·022	0·156
48	0·047	0·186
Reaction mixture: sulphur dioxide, 23 g.; 2N-hydrochloric acid, 50 g. Temperature, 110°		
20	—	0·077
24	—	0·089
34	0·010	0·117
36	0·021	0·134
48	0·029	0·115
	Sulphur removed by barium, g.	Sulphur not removed by barium, g.
Reaction mixture: sulphur dioxide, 23 g.; N-sulphuric acid, 50 g. Temperature, 110°		
20	0·070	—
30	0·092	—
36	0·139	—
41	0·148	0·0516
Reaction mixture: sulphur dioxide, 23 g.; 2N-sulphuric acid, 50 g. Temperature, 110°		
20	0·095	—
30	0·130	—
36	0·141	—
40	0·203	0·068

hydrolysis was calculated on the basis of the amino-nitrogen value obtained after heating a mixture of 10 g. of casein and 100 ml. of 5N-hydrochloric acid for 24 hours under reflux. The percentage of tryptophan was calculated on the basis of the nitrogen content of the sample multiplied by 6·38. Whole casein analysed in this way gave a tryptophan value of 1·26%.

The results reported in Table II show that sulphurous acid is a weak hydrolytic agent. It is noteworthy, however, that casein can be heated at 100° for 36 hours, under the conditions of this experiment, without appreciable destruction of tryptophan or humin formation.

This method was employed for the hydrolysis of fibrin, lactalbumin and ovalbumin. The

Table II

Hydrolysis of casein with sulphurous acid

Reaction time, h.	Degree of hydrolysis, %	Tryptophan content, %	Colour of hydrolysate
6	19	1·26	Colourless
12	26	1·24	,,
24	30	1·23	,,
30	33	1·24	,,
36	36	1·24	,,

Table III

Hydrolysis of fibrin, lactalbumin and ovalbumin with sulphurous acid

Protein	Degree of hydrolysis, %	Tryptophan content, %		Colour of hydrolysate
		Protein	Hydrolysate	
Fibrin (The British Drug Houses Ltd.)	37	2·48	2·48	Colourless
Lactalbumin (Champlain Milk Co.)	34	1·94	1·90	,,
Ovalbumin (General Chemical Co.)	36	1·30	1·26	,,

hydrolysates were analysed for amino-nitrogen, total nitrogen and tryptophan and the results obtained with a 24-hour heating period are shown in Table III.

Hydrolysis of proteins with mixtures of sulphurous acid and strong mineral acids

An attempt has been made to find conditions of hydrolysis that would give a higher degree of hydrolysis than was attained in the experiments described above, and would still not lead to tryptophan destruction or humin formation. In the following experiments, mixtures of 5 g. of casein, 50 ml. of mineral acid or water and 23 g. of sulphur dioxide were heated in sealed tubes with constant agitation. The products were analysed for amino-nitrogen, total nitrogen and tryptophan; results are shown in Tables IV and V.

Table IV

The effect of acid concentration and temperature on the degree of hydrolysis and tryptophan destruction

Reaction time, h.	Concn. of HCl	Temperature, °C	Degree of hydrolysis, %	Tryptophan content, %	Colour of hydrolysate
36	0	100	36	1·25	Colourless
20	1N	100	60	1·24	,,
12	1N	120	90	0·12	Dark brown
20	2N	100	70	1·00	Pale brown
20	5N	100	90	0·00	Very dark brown

Table V

Hydrolysis with a mixture of sulphurous acid and strong mineral acids at 100°

	Reaction time, h.	Degree of hydrolysis, %	Tryptophan content, %	Colour of hydrolysate
Reaction mixture: sulphur dioxide, 23 g.; N-hydrochloric acid, 50 ml.; casein, 5 g.				
	6	36	1·24	Colourless
	12	48	1·24	,,
	24	64	1·24	,,
	30	66	1·18	,,
	36	70	1·13	,,
Reaction mixture: sulphur dioxide, 23 g.; N-sulphuric acid, 50 ml.; casein, 5 g.				
	6	32	1·24	Colourless
	12	51	1·24	,,
	24	61	1·20	,,
	30	66	1·13	,,
	36	68	1·13	,,
Reaction mixture: sulphur dioxide, 23 g.; N-sulphuric acid, 50 ml.; casein, 5 g.				
	6	32	1·26	Colourless
	12	52	1·24	,,
	21	74	1·07	,,
	35	98	0·19	,,

Table VI

Protein	Degree of hydrolysis, %	Tryptophan content, %	
		Hydrolysate	Protein
Ovalbumin (General Chemical Co.)	69	1·25	1·30
Lactalbumin (Champlain Milk Co.)	70	1·75	1·94
Fibrin (The British Drug Houses Ltd.)	65	2·43	2·48

In order to test the hydrolytic and tryptophan-preserving effect of the sulphurous acid–N-sulphuric acid mixture on protein preparations other than casein, various animal proteins were heated in sealed tubes at 100° for 24 hours. The reaction mixtures consisted of 5 g. of proteins, 50 ml. of N-sulphuric acid and 23 g. of sulphur dioxide. The results are reported in Table VI.

In all the experiments described above, sufficient sulphur dioxide was used to ensure complete saturation of the aqueous phase with sulphur dioxide.[14, 15] In the following experiment the amount of sulphur dioxide was varied in order to find the minimal concentration that would prevent humin formation and tryptophan loss. The reaction mixtures, which consisted of 5 g. of casein, 50 ml. of N-sulphuric acid and sulphur dioxide, were heated for 24 hours in sealed tubes at 100°. The results reported in Table VII show clearly that a much larger quantity of sulphur dioxide was used in the previous experiments than was necessary to prevent tryptophan destruction.

Studies on sulphurous acid hydrolysates

Two casein hydrolysates were prepared by the use of sulphurous acid with (*a*) N-sulphuric acid, (*b*) N-hydrochloric acid. An outline of the procedure is given below.

Mineral acid (500 ml. of N-hydrochloric or N-sulphuric acid) was cooled to 0° and sufficient sulphur dioxide was dissolved in the acid to give effective tryptophan preservation. For sulphuric acid, 10 g. of sulphur dioxide/100 ml. was required, and for hydrochloric acid 15 g. Commercial casein (50 g.) was then added and the reaction mixture placed in a thick-walled, 900-ml. bottle. A rubber stopper was then wired firmly in place and the bottle was placed in a rocking device and heated for 24 hours at 100°. At the end of the heating period the bottle was cooled to 0° and opened. The sulphur dioxide was removed from the reaction mixture by evaporating the solution to half volume *in vacuo* at 40–50°.

The sulphate was precipitated from the hydrolysate by adding slowly, with stirring, slightly less than the calculated quantity of powdered barium hydroxide. The small amount of sulphate ion that still remained in the solution was removed by the addition of small quantities of hot, saturated barium hydroxide solution. The hydrolysate was considered to be free of barium ion and virtually free of sulphate ion when a small centrifuged aliquot of the mixture showed no turbidity when a drop of 1% sulphuric acid was added, and only a very slight turbidity when a drop of saturated barium hydroxide solution was added. The precipitate of barium sulphate was removed from the hydrolysate by centrifuging and washed twice by suspending it in a volume of water equal to one-third of the volume of the hydrolysate. The suspension was heated for 30 minutes at 80° and centrifuged. The filtrates and wash waters from these operations were then evaporated to dryness *in vacuo* at 60°.

The hydrochloric acid was removed from the hydrolysate by means of an anion-exchange resin (Nalcite SAR, Alchem Ltd., Burlington, Ontario). A sufficient quantity of the resin, in the sodium form, was added to the hydrolysate to raise the pH to 4; approximately 20 g.

Table VII

The hydrolysis of casein with N-sulphuric acid and various amounts of sulphur dioxide

Sulphur dioxide, g.	Degree of hydrolysis, %	Tryptophan content, %	Colour of hydrolysate
0·0	56	0·22	Dark brown
0·7	60	1·07	Pale yellow
2·9	60	1·11	Colourless
5·8	61	1·26	,,
7·3	60	1·26	,,
14·5	61	1·23	,,
21·8	60	1·24	,,

Table VIII

Analysis of casein hydrolysates

Hydrolysate	Degree of hydrolysis, %	Tryptophan content, %	Sulphurous acid calculated as sulphur dioxide, p.p.m.
Sulphurous acid–sulphuric acid	59	1·1	360
Sulphurous acid–hydrochloric acid	62	0·6	290

of moist resin was required per gram of protein in the original reaction mixture. The pH of the hydrolysate was then raised to pH 6 by the addition of dilute sodium hydroxide solution, and the hydrolysate was evaporated to dryness *in vacuo* at 40–50°. Table VIII shows the degree of hydrolysis and the tryptophan and sulphurous acid contents of these hydrolysates. The sulphurous acid was determined by the Monier-Williams method.[16] It will be noted that the sulphurous acid contents of the hydrolysates do not exceed the limit (500 p.p.m. in foods) set by the Canadian Food and Drug regulations.

The sulphurous acid–hydrochloric acid casein-hydrolysate possessed a pleasant meaty taste, free from any trace of the characteristic bitter taste of enzymic casein hydrolysates. The sulphurous acid–sulphuric acid hydrolysate had a meaty taste, but was slightly bitter.

Preliminary rat-feeding tests were based on the use of a control diet containing corn starch 70, casein 20, corn oil 5, bone char 2, brewer's yeast 2, sodium chloride 0·5 and vitamin-B_{12} supplement 0·6%. In the test groups the casein was replaced either by 20% enzymic casein hydrolysate (Lactamine AA, Champlain Chemicals, Stanbridge, Quebec) or by 20% sulphurous acid–sulphuric acid casein-hydrolysate (prepared above). Three groups of five male white rats, 20–28 days old, were fed for a period of 22 days; water and feed were supplied *ad lib.* The results of this experiment are shown in Table IX.

Table IX

Results of feeding trial

Nitrogen source	Average daily wt. gain, g.	Average daily food consumption, g.	Gain, g./100 g. of food consumption
Casein	1·58	8·9	17·8
H_2SO_3–H_2SO_4 casein-hydrolysate	0·89	7·6	10·7
Commercial enzymic hydrolysates	1·30	9·2	14·2

Discussion

The autoxidative properties of sulphurous acid were investigated with the aim of finding suitable conditions under which the acid might be used, either alone or in the presence of strong mineral acids, for the hydrolysis of proteins. The quantity of non-volatile sulphur-containing compounds formed in the reaction mixture was used as a measure of the amount of autoxidation.

The results reported in Table I demonstrate that the temperature, as well as the concentration of strong mineral acid, greatly affects the conversion of sulphur dioxide into the mixture of elemental sulphur, sulphuric acid and thionic acids. Hydrochloric acid and sulphuric acid seem to have about the same retarding effect on the autoxidation reaction, whereas casein showed no appreciable effect.

In experiments where sufficient sulphur dioxide was used to saturate the aqueous phase at 100°, proteins (casein, fibrin, lactalbumin and ovalbumin) were hydrolysed to the extent of only 30–37% in 24 hours at 100°. The hydrolysates were colourless and their tryptophan contents, as determined by the glyoxylic acid method, were approximately the same as those of the unhydrolysed proteins. Heating periods of more than 36 hours increased only slightly the degree of hydrolysis and gave sufficient conversion of the sulphur dioxide into thionic acids to interfere with the determination of tryptophan.

Addition of strong mineral acids to the mixture of protein, sulphur dioxide and water decreased the amount of conversion of sulphur dioxide into thionic acids and increased the degree of hydrolysis, but at the same time increased the tendency for humin formation and tryptophan destruction. Under conditions where there was little tryptophan destruction or humin formation, the degree of hydrolysis attained was not greater than 70%. The quantity

of sulphur dioxide required to give effective tryptophan retention in N-sulphuric acid, at 100°, was approximately 10 g./10 g. of casein in 100 ml. of sulphuric acid. Experiments with N-hydrochloric acid, under the same conditions, showed that approximately 15 g. of sulphur dioxide was required to give the same retention of tryptophan.

An attempt was made to devise a practical procedure for preparing protein hydrolysates suitable for high-protein therapy. The sulphur dioxide–sulphuric acid hydrolysate was not low in tryptophan, but it possessed a somewhat objectionable taste. This taste may have been due in part to the small quantity of sodium sulphate that the hydrolysate contained, or else to certain polypeptides that have been reported to possess an objectionable taste.[17] The sulphur dioxide–hydrochloric acid hydrolysate possessed a pleasant meaty taste, but its tryptophan content was low. Tryptophan determinations on the liquid hydrolysate, before treatment with the ion-exchange resin, showed that the tryptophan had not been destroyed during the hydrolysis. It may be concluded, therefore, that the tryptophan was held by the ion-exchange resin. Although the rat-feeding trial gave no conclusive results as to the nutritive value of the sulphur dioxide–sulphuric acid hydrolysate, it did show that the hydrolysate will support growth, and that it is not grossly toxic.

It is of interest to enquire into the mechanism of the tryptophan-preserving action of sulphurous acid. It might be that the effect of sulphurous acid is due solely to its reducing properties. Rigby's[4] work on the tryptophan-preserving action of nascent hydrogen shows clearly that proteins may be hydrolysed by acids, with little tryptophan destruction, if the reaction is performed under strong reducing conditions. This is supported by our observation that the decline in the tryptophan-preserving action of sulphurous acid with increasing concentration of hydrochloric acid runs parallel to its decline in reducing properties, as observed by Carter & Robinson.[18]

The action of sulphurous acid in preventing humin formation and tryptophan destruction may be attributed to its capacity for forming α-hydroxysulphonic acids with aldehydes. Although these acids are rather unstable and are decomposed by gentle heating in acid solution, it may be that, in the presence of a high concentration of sulphur dioxide, this decomposition does not take place. Hence the aldehydic groups may be unavailable for the reaction that causes humin formation and tryptophan destruction.

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