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SYNERGISTIC EFFECTS OF SOME COMPOUNDS RELATED TO 2-DIETHYLAMINOETHYL 2,2-DIPHENYL-n-PENTANOATE (SKF 525A) ON THE INSECTICIDAL ACTIVITY OF PYRETHRINS

By A. N. BATES, P. S. HEWLETT and C. J. LLOYD

A number of compounds related to SKF 525A were tested for synergism with pyrethrins, using Lesser Mealworm beetles, *Alphitobius laevigatus*, and houseflies, *Musca domestica*, as test insects. SKF 525A had previously been shown to be active, whereas 2,2-diphenyl-n-pentanoic acid and 2-diethylaminoethanol were inactive. Several active compounds resulted from esterification of 2-diethylaminoethanol with diphenylacetic acid and 1-substituted diphenylacetic acids. Most modifications of the alcoholic moiety of active 2-diethylaminoethyl esters destroyed activity. Active compounds resulted when a 2-diethylamino group was joined to a diphenylmethyl group through an ester, ketone or ether linkage, but the analogous amide was inactive. 2-Diethylaminoethyl piperonylate probably owed its activity to its piperonyl group. None of the compounds investigated approached piperonyl butoxide in synergistic activity.

Introduction

The ester 2-diethylaminoethyl 2,2-diphenyl-n-pentanoate (SKF 525A, see Table I) is well known to increase the effects on mammals of drugs of a number of different types.[1] Moreover, it has been shown to synergise the action of pyrethrins on certain insects.[2] In the work described here, a number of compounds related to SKF 525A have been tested for action as pyrethrin synergists on insects, in order to relate chemical structure to biological activity. Variation of the acid and alcohol components established sufficient conditions for synergism in esters. The effects of different linkages between the terminal moieties of the molecule were then examined.

Experimental

Methods of biological test

The test insects were Lesser Mealworm beetles, *Alphitobius laevigatus* (F.), and houseflies, *Musca domestica* L. They were reared by published methods,[3 4] except that the flies were fed on a sucrose solution after emergence. When dosed the beetles were 1–3 weeks old and the flies 3–5 days old.

The insects were dosed topically. A beetle was treated, without anaesthetisation, with 0·07 μl. of a solution of candidate synergist applied between the hind coxae by means of a micro-capillary tube;[5] 5–10 min. later it was treated with 0·025 μl. of a solution of pyrethrins applied dorsally on the neck by means of an air-pulse micro-drop applicator.[3] The pyrethrins were dissolved in Shell Risella oil 17 at a concentration of 0·5% w/v. Most of the candidate synergists were dissolved in odourless kerosene at 10% w/v. However, owing to low solubility in kerosene, four compounds (2,2-diphenyl-n-pentanoic acid, diphenylacetic acid, lignocaine and compound III of Table I) were dissolved at 6% w/v in odourless kerosene containing 40% v/v of cyclohexanone, and three compounds (Marsilid, JB 516 and compound XIII of Table I) at 10% w/v in cyclohexanone alone. Dosage with pyrethrins without synergist was done comparably, with appropriate solvent on the hind coxae and pyrethrin solution on the neck.

A fly was dosed, under brief carbon dioxide anaesthesia, with a solution containing both pyrethrins and the candidate synergist by means of a micro-capillary tube;[5] it was treated dorsally between the scutum and scutellum, a male with 0·05 μl. and a female with 0·10 μl. In most of the experiments with flies, the solvent was odourless kerosene; the concentration of pyrethrins varied from 1·0 to 1·8% w/v according to the test, and the concentration of the candidate synergist was 10 times that of the pyrethrins. However, compounds III, XIII and lignocaine were used, with pyrethrins, in solution in cyclohexanone, the concentration of the compound being 10% w/v. Dosage with pyrethrins without synergist was done comparably.

The beetles were kept at 25° after dosage, the flies at 27°. Knock-down in the groups of beetles was determined at 4 days after dosage, and mortality at 6 and 9 days. Knock-down

and mortality of flies were determined 24 h. after dosage. At the doses used the solvents alone were non-toxic to the insects, as were all the candidate synergists.

Chemical preparations

The physical properties of the compounds prepared are listed in Table II, together with details of microanalyses.

Esters (Compounds II-V, VII-XI).—All compounds containing an ester linkage were prepared by condensation of the appropriate acid chloride and alcohol. Neutral esters were purified by distillation of the reaction products *in vacuo*, and basic esters by precipitation and recrystallisation of the hydrochlorides.

Ethyl 2,2-diphenyl-n-pentanoate (XII).—Ethyl diphenylacetate (6·6 g.) and sodamide (1 g.) in anhydrous benzene (30 ml.) were heated under reflux with stirring for 22 h. After the reaction mixture had been cooled, propyl iodide (2·7 ml.) was added and the mixture was again heated under reflux with stirring for a further 24 h. The solution was filtered and fractionated *in vacuo*. Ethyl 2,2-diphenyl-n-pentanoate (2 g.) was obtained as a pale green oil.

Ethyl 2,2-diphenyl-n-pentanoate was hydrolysed with ethanolic potash for 72 hours under reflux. The solution was acidified and extracted with diethyl ether. The ethereal extract yielded colourless crystals, m.p. 153–155°, of 2,2-diphenyl-n-pentanoic acid.[6] (Found: C, 80·0; H, 7·1. $C_{17}H_{18}O_2$ requires C, 80·3; H, 7·1%.)

N-(*2-diethylaminoethyl*) *diphenylacetamide* (XIII) was prepared by condensation of diphenylacetyl chloride with diethylamino ethylamine, according to the method of Mndzhoyan.[7]

2-Diethylaminoethyl diphenylmethyl ketone (XIV) was prepared by the condensation of 1,1-diphenylacetone, formaldehyde and diethylamine in acid medium, according to the method of Sprague & Schultz.[8]

2-Diethylaminoethyl diphenylmethyl ether (XV) was prepared by the condensation of 2-diethylaminoethyl chloride with benzhydrol in alkaline medium, according to the method of Forberg.[9]

4-Diethylamino-1,1-diphenylbutane (XVI) was prepared by the condensation of 3-chloropropyl-*N*-diethylamine with the sodium derivative of diphenylmethane in an atmosphere of nitrogen, according to the method of Benoit *et al.*[10]

Results

Table I lists the more important of the compounds tested and indicates their effects on the toxicity of pyrethrins. Other compounds, less closely related chemically to SKF 525A, were also tested, namely: the alcohol and acid components of SKF 525A, 2-diethylaminoethanol and 2,2-diphenyl-n-pentanoic acid; diphenyl acetic acid; diphenylpropylacetonitrile; (±)-6-dimethylamino-4,4-diphenylheptan-3-one (methadone); and ω-diethylamino-2,6-dimethylacetanilide (lignocaine). Of these, diphenylpropyl-acetonitrile was feebly synergistic for houseflies, and lignocaine feebly synergistic for both species; otherwise these compounds were inactive. Two inhibitors of mammalian monoamine oxidase, *N*-isonicotinoyl-*N'*-isopropylhydrazine (Marsilid) and 2-phenyl isopropylhydrazine (JB 516), were tested on the beetles only, and the former was feebly active.

Discussion

Table I gives results for the presence or absence of synergism, with a minor gradation provided by distinguishing between marked and slight synergism. However, a few additional comparisons were made, the results of which are indicated in the course of this discussion. In the Table there are relatively few discrepancies between the results for beetles and those for flies. Where the results differed, the compound was active on the beetles but not on the flies.

Compounds I-VII in Table I were different esters of 2-diethylaminoethanol. The activity of compound III shows that two unsubstituted phenyl groups in an acetic acid moiety were sufficient to give a synergistic ester, although compound III was less active than SKF 525A. The inactivity of compounds IV and V indicates that a single unsubstituted phenyl group in the

Table I

Synergistic activity of compounds related to SKF 525A

Number	Candidate synergist* Name	Formula	Synergistic activity† *A. laevigatus*	*M. domestica*
I	2-Diethylaminoethyl 2,2-diphenyl-n-pentanoate (SKF 525A)	$C_2H_7{\cdot}C(C_6H_5)_2{\cdot}CO{\cdot}O{\cdot}CH_2{\cdot}CH_2{\cdot}N(C_2H_5)_2$	+	+
II	2-Diethylaminoethyl 2,2-diphenylbutyrate	$C_2H_5{\cdot}C(C_6H_5)_2{\cdot}CO{\cdot}O{\cdot}CH_2{\cdot}CH_2{\cdot}N(C_2H_5)_2$	+	+
III	2-Diethylaminoethyl diphenylacetate (Trasentin)	$(C_6H_5)_2CH{\cdot}CO{\cdot}O{\cdot}CH_2{\cdot}CH_2{\cdot}N(C_2H_5)_2$	+	+
IV	2-Diethylaminoethyl phenylacetate	$C_6H_5{\cdot}CH_2{\cdot}CO{\cdot}O{\cdot}CH_2{\cdot}CH_2{\cdot}N(C_2H_5)_2$	o	o
V	2-Diethylaminoethyl benzoate	$C_6H_5{\cdot}CO{\cdot}O{\cdot}CH_2{\cdot}CH_2{\cdot}N(C_2H_5)_2$	o	o
VI	2-Diethylaminoethyl 2,2,3-triphenylpropionate	$C_6H_5{\cdot}CH_2{\cdot}C(C_6H_5)_2{\cdot}CO{\cdot}O{\cdot}CH_2{\cdot}CH_2{\cdot}N(C_2H_5)_2$	+	o
VII	2-Diethylaminoethyl piperonylate	$CH_2O_2C_6H_3{\cdot}CO{\cdot}O{\cdot}CH_2{\cdot}CH_2{\cdot}N(C_2H_5)_2$	+	+
VIII	2-Di-n-butylaminoethyl diphenylacetate	$(C_6H_5)_2CH{\cdot}CO{\cdot}O{\cdot}CH_2{\cdot}CH_2{\cdot}N(C_4H_9)_2$	+	o
IX	2-Dimethylaminoethyl diphenylacetate	$(C_6H_5)_2CH{\cdot}CO{\cdot}O{\cdot}CH_2{\cdot}CH_2{\cdot}N(CH_3)_2$	o	o
X	Isopentyl diphenylacetate	$(C_6H_5)_2CH{\cdot}CO{\cdot}OC_5H_{11}$	o	o
XI	Isopentyl 2,2-diphenylbutyrate	$C_2H_5{\cdot}C(C_6H_5)_2{\cdot}CO{\cdot}OC_5H_{11}$	o	o
XII	Ethyl 2,2-diphenyl-n-pentanoate	$C_3H_7{\cdot}C(C_6H_5)_2{\cdot}CO{\cdot}OC_2H_5$	o	(not tested)
XIII	*N*-(2-Diethylaminoethyl)-diphenylacetamide	$(C_6H_5)_2CH{\cdot}CO{\cdot}NH{\cdot}CH_2{\cdot}CH_2{\cdot}N(C_2H_5)_2$	o	o
XIV	2-Diethylaminoethyl diphenylmethyl ketone	$(C_6H_5)_2CH{\cdot}CO{\cdot}CH_2{\cdot}CH_2{\cdot}N(C_2H_5)_2$	+	(+)
XV	2-Diethylaminoethyl diphenylmethyl ether	$(C_6H_5)_2CH{\cdot}O{\cdot}CH_2{\cdot}CH_2{\cdot}N(C_2H_5)_2$	+	+
XVI	4-Diethylamino-1,1-diphenylbutane	$(C_6H_5)_2CH{\cdot}(CH_2)_2{\cdot}N(C_2H_5)_2$	+	

* All dialkylamino derivatives were tested as the free base
† +, Marked synergism; (+), slight synergism; o, no effect

Table II

Chemical data on candidate synergists

Compound Number	Physical constants Boiling point or melting point	n_D	Analyses Carbon % Found	Carbon % Required	Hydrogen % Found	Hydrogen % Required	Nitrogen % Found	Nitrogen % Required
II*	124–126°		70·5	70·3	8·3	8·1		
III*	113–115°		69·0	69·1	7·5	7·5		
IV	104–109°/0·1 mm.	$1{\cdot}4947^{22°}$	70·8	71·4	9·1	9·0	6·1	6·0
V*	122–124°		60·9	60·5	7·8	7·8		
VII*	136–138°		55·7	55·7	6·4	6·6	4·4	4·6
VIII*	83–84°		71·3	71·4	8·2	8·4		
IX*	159–161°		67·9	67·6	7·2	6·9		
X	91–93°/0·5 mm.	$1{\cdot}5350^{24°}$	81·1	80·9	7·7	7·8		
XI	148°/0·3 mm.	$1{\cdot}5340^{25°}$	81·5	81·3	8·6	8·4		
XII	75–82°/0·2 mm.	$1{\cdot}5449^{27°}$	81·1	80·9	8·3	7·8		
XIII	93–95°		76·9	77·4	8·4	8·4	9·5	9·0
XIV*	140–142°		71·8	72·4	7·8	7·9	4·2	4·2
XV	126–127°/0·1 mm.	$1{\cdot}5359^{26°}$	80·4	80·5	9·0	8·9		
XVI	163–164°/1 mm.	$1{\cdot}5395^{22°}$	84·2	85·4	9·6	9·7	5·0	5·0

* Results for these compounds refer to the hydrochlorides

acid does not confer activity. Compound VI, with three phenyl groups in the acid, was active on the beetles—slightly more so, in fact, than SKF 525A—but was inactive on flies. The activity of the piperonylic ester (VII), compared with the inactivity of compound V, suggests that compound VII owes its activity to the piperonyl group, especially since many piperonyl compounds synergise the action of pyrethrins on insects; compound VII, like SKF 525A, was much less active than piperonyl butoxide, a pyrethrin synergist used in practical insect control.[11]

The results with compounds VIII–XII indicate that the alcoholic portion of an active ester can be varied but little if activity is to be retained. Whereas the 2-diethylamino ester of diphenylacetic acid (III) was active, the 2-dimethylamino ester (IX) was inactive, and the 2-di-n-butylamino ester was active only on the beetles. Comparison of the inactivity of compounds X, XI and XII with the activity of compounds III, II and I, respectively, indicates that an aliphatic alcohol does not confer activity.

Compounds III and XIII–XVI form a series in which a 2-diethylamino group was joined to a diphenylmethyl group by different linkages. Ester (III), ketone (XIV) and ether (XV) linkages gave active compounds; a simple hydrocarbon linkage (XVI) gave a compound active on beetles but inactive on flies; an amide linkage (XIII) gave an inactive compound, but this is relatively insoluble in lipophilic solvents, and may not pass readily through insect cuticle.

To sum up, active compounds were obtained when a 2-diethylamino moiety was joined to a diphenylmethyl moiety through an ester, ketone or ether linkage. For the ester linkage at least, the latter moiety could be replaced by a 1,1-diphenyl-n-butyl group or a 1,1-diphenylpropyl group (compounds I and II).

It was found previously[2] that both piperonyl butoxide and SKF 525A synergised the action of pyrethrins on insects of the species used here, but both antagonised the action of malathion. Antagonism between piperonyl butoxide and malathion has been noted by other workers using houseflies[12,13] and the mosquito *Anopheles stephensi*.[14] Recently Abdallah[15] has suggested that in the work just referred to the two compounds may have antagonised malathion merely by retarding its penetration into the insect. He observed antagonism when TOCP was applied topically on the same part of the housefly as parathion or paraoxon, but synergism when applied to a different part. However, in work with beetles[2] and in some of that on houseflies,[13] the doses of malathion and of its antagonist were put respectively on to different parts of the surface of the insect. Under these circumstances the antagonism is likely to be a biochemical phenomenon—perhaps due to depression by the antagonist of the biological oxidation of malathion to the more toxic malaoxon.[16] Similarly, synergists for pyrethrins may act by depressing the metabolism (probably oxidative, at least in part) of pyrethrins to products not yet identified.

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THE SEARCH FOR A VETERINARY INSECTICIDE
I.—Sulphonamides and Disulphonamides active against Sheep Blowfly

By D. GREENWOOD and I. R. HARRISON

Laboratory and field test methods are described for assessing the activity of chemicals for the control of sheep blowfly *Lucilia sericata* (Meig.). The essential properties for a sheep blowfly insecticide are discussed. Screening results for several series of sulphonamides and disulphonamides, with and without *N*-substituents, are presented. Although some disulphonamides showed high activity, none had the necessary persistence for use in a sheep-dip.

Introduction

The sheep blowfly is the most important external parasite of sheep in most of the sheep-rearing areas of the world. During its active period, the female lays eggs on sheep fleece and, unless the sheep has been treated with a suitable insecticide, the newly hatched larvae move down the wool staple and start to feed upon the skin, causing a lesion (commonly called a ' strike '). This extends in size and becomes very attractive to other species of blowfly, which lay their eggs on the site of the initial attack. Secondary infestations may result. Before the use of modern insecticides, sheep blowfly was responsible for high sheep mortalities.

To be of practical utility in the prevention of sheep blowfly ' strike ', a compound must possess three basic properties:

(1) It must possess a high level of insecticidal activity against the larval stage of the sheep blowfly, as it is not possible during the short period of oviposition so to affect the adult fly as to prevent eggs being laid on the fleece.

(2) It must persist on the fleece maintaining its activity for a long period, preferably at least 12 weeks, as it is not practicable to round up sheep at frequent intervals for insecticidal treatment.

(3) It must be safe to sheep and humans, i.e., of low mammalian toxicity. Sheep are normally treated by either dipping or spraying, and during this treatment the farmworkers cannot avoid coming into contact with the chemical being used.

A number of compounds, including D.D.T. and B.H.C., have been used to protect sheep from blowfly strike, but the most successful have been the chlorinated bicycloheptene derivatives, for example, dieldrin and aldrin. Both these compounds are extremely toxic to sheep blowfly larvae and protect sheep from strike for periods larger than 12 weeks. However, for two reasons, these compounds are now used less frequently. Firstly, the phenomenon of resistance has occurred; strains of sheep blowfly have evolved, the larvae of which are not susceptible to dieldrin, aldrin or other chlorinated hydrocarbon insecticides. Secondly, it has been realised that these chemicals are stored in the fat of treated animals and, because of the resulting toxicity hazard to humans, the Health Authorities of many countries, including U.S.A., Australia, New Zealand and Great Britain, have banned their use.

Attention has been focused upon the organophosphorus compounds, a few of which are now used commercially for the prevention of sheep blowfly strike. Diazinon exhibits extremely high insecticidal activity towards the larvae of sheep blowfly, it is of low toxicity to humans, and persists on sheep fleece for several weeks. Although in this last respect it is inferior to dieldrin, diazinon has found widespread use. Many other organophosphorus compounds possess high insecticidal activity and relatively low mammalian toxicity, but are not used in the treatment of sheep because of lack of persistence. Although no cases of diazinon-resistant sheep blowfly larvae have been reported, there have been reports of the evolution of houseflies and cattle ticks which are resistant to organophosphorus compounds. Unfortunately, insects become resistant to groups of chemical compounds such as chlorinated hydrocarbons or organophosphorus compounds, and not merely to specific compounds.

For these reasons, the search for new compounds to control sheep blowfly strike has concentrated on compounds of very different chemical groups. In the present work, many aromatic sulphonamides and disulphonamides have been synthesised and tested. The effect of variations

of the nuclear substituents has been investigated, and several disulphonamides showing high insecticidal activity have been found. These compounds are soluble in water, however, especially at alkaline pH, and as alkaline conditions are usual in sheep-fleece, long persistence could not be expected. Many derivatives bearing a wide variety of *N*-substituents were therefore prepared, as it was hoped that the completely substituted derivatives, being insoluble in alkali, would prove to be persistent.

The insecticidal activity of disulphonamides has not previously been reported. Disulphonamides inhibit carbonic anhydrase, and are used as diuretics.[1] There have been several reports of the activities of monosulphonamides as insecticides. Runge & El-Hewehi[2] described the screening of *N*-substituted 2,5-dichloro- and 2,4,5-trichloro-benzenesulphonamides against the clothes moth (*Tineola bisselliella*), *Anthrenus vorax* and *Attagenius piceus*. Stota[3] mentioned similar compounds active against housefly (*Musca domestica*) and clothes moth, and Misra & Asthana[4] described the activity of *N*-cyanoethylated sulphonamides. Sulphonanilides bearing trifluoromethyl substituents have been claimed[5] for the control of textile pests.

Experimental

(1) *Synthesis of compounds*

Most of the analyses of the new compounds are recorded in the Tables. Where this is not convenient, for example when different elements were estimated, the analyses are given in the text.

The disulphonamides listed in Table I were usually prepared by chlorosulphonation of the appropriate hydrocarbons with excess of chlorosulphonic acid at 120–160° until evolution of hydrogen chloride ceased, isolation of the crude di(sulphonyl chlorides) by addition to ice and water, and reaction with excess of liquid ammonia. They were purified by extraction into alkali, acidification, and recrystallisation from a suitable solvent. This is essentially the method described by Boggiano *et al.*[1]

Propylbenzene-2,4-disulphonamide had m.p. 177–179° (Found: N,9·8. $C_9H_{14}N_2O_4S_2$ requires N, 10·1%). The method failed with butylbenzene, t-butylbenzene and hexylbenzene, apparently due to attack of the chlorosulphonic acid on the side-chains.

The disulphonamides from mesitylene,[6] anisole,[7] *m*-chloroaniline[8] and *m*-trifluoromethylaniline[8] were prepared by variations of this method. Acetylation of the last product gave 1-acetamido-3-trifluoromethylbenzene-4,6-disulphonamide.[9] The *disulphonamide* from 1,3,5-trimethyl-2-nitrobenzene-4,6-di(sulphonyl chloride)[6] had m.p. 285–287° (Found: N,12·5; S,19·8. $C_9H_{13}N_3O_6S_2$ requires N, 13·0; S, 19·8%).

Oxidation of toluene-2,4-disulphonamide gave carboxybenzenedisulphonamide,[10] which yielded a *methyl ester*, m.p. 197–198° (Found: C, 33·1; H, 3·1. $C_8H_{10}N_2O_6S_2$ requires C, 32·65; H, 3·4%). Reactions of the ester with ammonia failed to give the amide, as cyclisation took place yielding 6-sulphamoylbenzo[*d*]-1,2-thiazolin-3-one-1,1-dioxide (6-sulphamoylsaccharin). Attempts to form cyanobenzenedisulphonamides by chlorosulphonations of aryl cyanides or by diazotisations of aminobenzenedisulphonamides also failed. A reaction between bromobenzene-2,4-disulphonamide and cuprous cyanide in dimethylformamide, a recently-described modification[11] of the Rosenmund–von Braun conditions, gave a product, m.p. 290–297° (decomp.), the infra-red spectrum (Mr. W. Brown) of which suggested that it had the cyclised structure, *6-sulphamoylbenzo*-[d]-*1,2-thiazolin-3-imine-1,1-dioxide* (3-imino-6-sulphamoylsaccharin) (Found: N, 15·8. $C_7H_7N_3O_4S_2$ requires N, 16·1%).

Bromobenzene-2,4-disulphonamide interacted with the sodium salts of thiols when refluxed in ethanol for 1–4 days. *Butylthiobenzene-2,4-disulphonamide*, m.p. 202–204° (Found: S, 28·5. $C_{10}H_{16}N_2O_4S_3$ requires S, 29·6%) gave a *sulphone*, m.p. 220–222° (Found: S, 27·1. $C_{10}H_{16}N_2O_6S_3$ requires S, 27·0%). p-*Chlorophenylthiobenzene-2,4-disulphonamide*, m.p. 217–218° (Found: Cl, 9·2; S, 25·2. $C_{12}H_{11}ClN_2O_4S_3$ requires Cl, 9·4; S, 25·4%) and the corresponding *sulphone*, m.p. 274° (Found: Cl, 8·9; S, 23·4. $C_{12}H_{11}ClN_2O_6S_3$ requires Cl, 8·65; S, 23·4%), and p-*chlorobenzylthiobenzene-2,4-disulphonamide*, m.p. 208–213° (Found: Cl, 8·9; S, 24·7. $C_{13}H_{13}ClN_2O_4S_3$ requires Cl, 9·0; S, 24·5%) and its *sulphone*, m.p. 307° (decomp.) (Found: Cl, 8·8; S, 22·6. $C_{13}H_{13}ClN_2O_6S_3$ requires Cl, 8·4; S, 22·6%) were prepared similarly.

Table I

Substituted benzene-1,3-disulphonamides: % mortality of sheep blowfly larvae and persistence

R.D. No.	Substituents	Concentration, % 0·05	0·025	0·0125	0·006	0·003	Persistence*
14527	—	100	100	0	0	0	—
12835	4-Me	100	100	100	100	100	0
15386	4,6-Me_2	100	100	100	100	47	1
15995	2,4,6-Me_3	0	0	0	0	0	—
15871	4-Et	100	100	88	0	0	—
15926	4-Pr	100	100	100	68	45	0
13671	4-Cl	100	100	100	78	51	1
15443	4,6-Cl_2	0	0	0	0	0	—
15647	4-Br	100	100	31	0	0	—
15873	4-F	100	100	100	80	59	0
15383	4-Cl-6-Me	100	100	100	82	40	—
13666	4-NH_2-6-Cl	44	23	0	0	0	—
13667	4-NH_2-6-CF_3	84	0	0	0	0	—
15538	4-AcNH-6-CF_3	0	0	0	0	0	—
16050	2,4,6-Me_3-5-NO_2	0	0	0	0	0	—
15649	4-MeO	0	0	0	0	0	—
15532	4-COOH	0	0	0	0	0	—
15535	4-COOMe	0	0	0	0	0	—
15826	4-CN (cyclised)†	0	0	0	0	0	—
16120	4-*p*-Cl·C_6H_4S	70	0	0	0	0	—
16122	4-*p*-Cl·C_6H_4·CH_2S	0	0	0	0	0	—
16124	4-BuS	100	43	0	0	0	—
16121	4-*p*-ClC_6H_4·SO_2	90	10	0	0	0	—
16123	4-*p*-ClC_6H_4·CH_2·SO_2	0	0	0	0	0	—
16129	4-BuSO_2	38	0	0	0	0	—

* — no test 0 = no persistence 1 = persisted for 1 week

† 6-Sulphamoylbenzo[*d*]-1,2-thiazolin-3-imine-1,1-dioxide

The known disulphonamides had melting points in approximate agreement with the literature values, as had the monosulphonamides in Table II, which were acquired from commercial sources or prepared by standard methods as described in the literature.

The following examples illustrate the methods used for the introduction of *N*-substituents.

Toluene-2,4-bis-sulphon-N-*ethylamide.*—Toluene-2,4-di(sulphonyl chloride) (10 g.) in dry benzene (50 c.c.) was added during 30 min. to ethylamine (10 g., excess) in dry benzene (50 c.c.) at 20–25°. Next day, the solution was washed with dilute acid, and then extracted several times with dilute alkali. Acidification gave the *product*, m.p. 84–90°. Two recrystallisations from trichloroethylene gave 7·4 g. (70%) m.p. 92–94°.

Other products from primary aliphatic amines were prepared similarly. The products from secondary aliphatic amines, insoluble in alkali, were isolated by removal of the benzene and recrystallisation of the residue.

The reactions with aromatic amines were carried out using only two equivalents of the amines and two equivalents of pyridine. The products were isolated by similar methods.

Toluene-2,4-bis-sulphon-N-*methoxy*-N-*methylamide.*—*N*-Methoxy-methylamine hydrochloride (4·9 g.) was added to dry pyridine (60 c.c.), and the solution was treated with toluene-2,4-di(sulphonyl chloride) (7·25 g.) as above. After 3 days, addition to excess of dilute acid, filtration and recrystallisation from ethanol gave the *product* (6·4 g., 76%).

Toluene-2,4-bis-sulphon-N-*benzyl*-N-*methylamide.*—To a solution of sodium (0·6 g.) in ethanol (30 c.c.) was added toluene-2,4-bis-sulphon-*N*-methylamide (3·0 g.) followed by benzyl chloride (3·0 g.). The solution was refluxed for 2 h., cooled and added to dilute alkali. The *product* (3·9 g., 79%) was recrystallised from ethanol.

Toluene-2,4-bis-sulphon-N-*cyanomethyl*-N-*methylamide.*—Toluene-2,4-bis-sulphon-*N*-methylamide (10·4 g.), potassium carbonate (10·5 g.), chloroacetonitrile (6·0 g.) and dry acetone (100 c.c.) were refluxed for 8 h. Filtration, removal of most of the acetone, and trituration with ice-cold alkali left a dark sticky material. Three crystallisations from ethanol gave the *product* (3·6 g., 37%).

Table II

Benzenesulphonamides: % mortality of sheep blowfly larvae

R.D. No.	Substituents	Concentration, %				
		0·05	0·025	0·0125	0·006	0·003
13805	—	70	0	0	0	0
13675	4-Me	50	0	0	0	0
15502	2,4,6-Me_3	0	0	0	0	0
15648	4-t-Bu	0	0	0	0	0
13682	4-Cl	100	100	42	0	0
16891	3-CF_3	100	100	52	44	26
7735	2-NO_2	0	0	0	0	0
6301	3-NO_2	20	0	0	0	0
14378	2-Cl-5-NO_2	0	0	0	0	0
6193	2-MeO	0	0	0	0	0
15444	2-CN	0	0	0	0	0
15517	4-MeS	86	0	0	0	0
15531	4-$MeSO_2$	100	100	65	0	0

Toluene-2,4-bis-sulphon-N-*acetylamide.*—Toluene-2,4-disulphonamide (5·15 g.) and acetic anhydride (6·3 c.c.) were heated at 140° for 4½ h., and then added to cold water. The *product* (4·3 g., 63%) recrystallised from ethyl acetate or ethanol. (Found: C, 39·9; H, 4·6. $C_{11}H_{14}N_2O_6S_2$ requires C, 39·5; H, 4·2%).

Toluene-2,4-bis-sulphon-N-*acetyl*-N-*methylamide* was obtained in small yield from the disodium salt of toluene-2,4-bis-sulphon-*N*-methylamide and acetyl chloride in toluene. The disodium salt and toluene-*p*-sulphonyl chloride, heated at 140° for 5 h., gave *toluene-2,4-bis-sulphon*-N-*methyl*-N-p-*toluenesulphonamide* (20%). The disodium salt and toluene-2,4-di(sulphonyl chloride) gave a *polymeric product* [Found: N, 5·6. $(C_8H_9NO_4S_2)_x$ requires N, 5·7%].

Toluene-2,4-bis-sulphon-N-p-*chlorophenyl*-N-*ethoxycarbonylamide.*—Toluene-2,4-bis-sulphon-*N-p*-chlorophenylamide (4·7 g.), potassium carbonate (3 g.), ethyl chloroformate (2·6 g.) and dry acetone (30 c.c.) were refluxed for 6 h. Filtration and removal of the acetone left the *product* (5·0 g., 81%) (Found: Cl, 11·75. $C_{25}H_{24}Cl_2N_2O_8S_2$ requires Cl, 11·5%), which was recrystallised from ethanol. *Toluene-2,4-bis-sulphon*-N-*ethoxycarbonyl*-N-*methylamide* and *toluene*-p-*sulphon*-N-*ethoxycarbonyl*-N-*methylamide*, prepared similarly, were obtained as viscous oils.

When α-naphthyl chloroformate was used in similar reactions, the products were contaminated with α-naphthyl carbonate. However, when the *N*-α-naphthyloxycarbonylsulphonamides were prepared from α-naphthyl chloroformate and the sodium salts of the sulphonamides in toluene, this impurity was not observed.

The *N*-substituted derivatives of toluene-*p*-sulphonamide listed in Table IV were obtained by similar methods. The melting points recorded for the known compounds are in approximate agreement with the literature values.

p-*Chlorobenzenesulphon*-N-*methyl*-N-α-*naphthyloxycarbonylamide*, m.p. 95–97° (Found: S, 8·5; $C_{18}H_{14}ClNO_4S$ requires S, 8·5%) and p-*chlorobenzenesulphon*-N-3,4-*dichlorophenyl*-N-*ethoxycarbonylamide*, m.p. 130° (Found: Cl, 26·3; S, 7·9; $C_{15}H_{12}Cl_3NO_4S$ requires Cl, 26·1; S, 7·8%) were also prepared.

(2) *Rearing of sheep blowfly and laboratory test methods*

Dorman *et al.*[16] and Cammiade[17] gave details of a technique for the breeding of sheep blowfly. The methods described in these two papers were modified slightly because some difficulty was experienced in obtaining sufficient numbers of fertile eggs throughout the year. The adult flies were kept in cages at a constant temperature of 25° ±1° and a relative humidity of 55 ±2%, with continuous illumination from an overhead strip light. They were fed on cubed sugar and water given by means of a moist cotton wool pad. Approximately 1000 flies were kept in a cage 20 in. high, 12 in. wide and 12 in. deep. Fresh liver was placed in the cage each day and the female flies laid their eggs on the liver 2–3 days after their first protein feed. Newly-hatched larvae used for testing purposes were removed from the liver by shaking in a beaker of water. The larvae sank to the bottom and could be pipetted out.

The preliminary test was carried out with solutions in acetone at 0·25% and 0·05% using two replicates per concentration. Cotton wool dental bungs were placed in 3 in. × ½ in. tubes and impregnated with 0·5 c.c. of the solution, after which they were allowed to dry over a 24-h. period. When evaporation was complete, 1 c.c. of sheep serum was run into each tube and approximately 20 newly-hatched larvae were introduced. The tubes were then sealed with a loose cotton wool bung and placed under a strip light to ensure that the larvae went down to the bung. The percentage mortality after 24 h. was recorded.

Any compounds showing 100% mortality at 0·25% and 0·05% were retested in the same manner but with three replicates each at concentrations of 0·05%, 0·025%, 0·0125%, 0·00625% and 0·003125%.

(3) *Testing on sheep*

Compounds which showed high activity at 0·0125% are tested on sheep, since conditions of persistence on sheep fleece cannot be simulated satisfactorily in the laboratory. The compounds were dissolved in either acetone or xylene and then dispersed in water using Ethylan B.V. and Arylan C.A. Five hundred c.c. of spray were applied to each sheep using a precision sprayer operating at a constant pressure of 40 p.s.i. This amount of insecticide is sufficient to soak the wool completely down to the skin over a wide strip in the middle of the back from the neck to the top of the tail. The sheep were sprayed at a range of concentrations from 0·1% to 0·005%. With each group of compounds sprayed, a standard dieldrin spray was applied at 0·05% for comparison. Dieldrin will persist for 16–18 weeks.

After treatment, the sheep were returned to the outdoor flock as soon as the spray was dry; they were brought in for examination at weekly intervals. Firstly, a wool sample was assayed in the laboratory using sheep blowfly larvae.[18] From the percentage kill of larvae exposed to this treated wool can be calculated the amount of chemical still remaining. Secondly, the sheep were implanted with first instar larvae of the sheep blowfly. If sufficient chemical still remained in the fleece, the larvae were killed before they could start feeding on the surface of the skin. The sheep were examined 24 h. later to see the result of the implant. The implant should not be positive until the wool assay percentage mortality is less than approximately 20%, but variations of insecticide take-up over the surface occur so that in some cases the implant may be positive whilst the wool sample still shows some chemical is present.

Results

Table I shows the activity of the nuclear substituted 1,3-disulphonamides against sheep blowfly larvae and the persistence on sheep fleece. Table II lists similar monosulphonamides for comparison and Tables III and IV give the results for *N*-substituted disulphonamides and monosulphonamides respectively. As the persistence test depends on a biological assessment, no figure can be given for inactive compounds.

Discussion of results

Table I shows the extremely high activities of some of the disulphonamides substituted only in the nucleus, and demonstrates the large variations of insecticidal activity caused by the introduction of different nuclear substituents. The most active compounds were those containing one or two methyl groups. In view of this, the complete lack of activity when three methyl groups were introduced was very unexpected. Activity was retained in the monoalkyl derivatives as the substituent was varied through methyl, ethyl and propyl.

A halogen substituent (fluoro, chloro, and to a lesser extent bromo) also enhanced the activity of the unsubstituted benzene-1,3-disulphonamide, and in this series the activity was lost completely in the dichloro compound. The compound bearing a chloro and a methyl substituent was, however, very active. All the other substituents introduced gave lower or even no activity.

In all cases where a direct comparison is possible, the monosulphonamides listed in Table II were much less active than the corresponding disulphonamides.

Unfortunately, none of the active compounds showed any persistence on sheep-fleece, and it was in an attempt to remedy this defect that the *N*-substituted derivatives in Tables III and IV

Table III

NN'-Substituted toluene-2,4-disulphonamides [$CH_3 \cdot C_6H_3(SO_2 \cdot NRR')_2$]

R.D. No.	R	R'	M.p., °c	Found, % N	Found, % S	Formula	Required, % N	Required, % S	% Mortality to sheep blowfly larvae at concn. 0·25%	0·05%	0·025%	0·0125%
14529	Me	H	125—127	10·3		$C_9H_{14}N_2O_4S_2$	10·1		100	90	24	0
14533	Et	H	92—94	9·45		$C_{11}H_{18}N_2O_4S_2$	9·15		100	95	36	10
14534	Allyl	H	56—61	8·9	19·15	$C_{13}H_{18}N_2O_4S_2$	8·5	19·4	100	94	74	0
14532	Bu	H	50—55		17·2	$C_{15}H_{26}N_2O_4S_2$		17·7	84	0	0	0
14574	s-Bu	H	137—138		17·6	$C_{15}H_{26}N_2O_4S_2$		17·7	23	0	0	0
14573	t-Bu	H	221—222		17·5	$C_{15}H_{26}N_2O_4S_2$		17·7	0	0	0	0
14568	Dodecyl	H	87—94[a]		10·2	$C_{31}H_{58}N_2O_4S_2$		10·9	0	0	0	0
14535	Ph	H	189—190[b]			$C_{19}H_{18}N_2O_4S_2$			0	0	0	0
14566	4-ClC_6H_4	H	200—201		13·3	$C_{19}H_{16}Cl_2N_2O_4S_2$		13·6	0	0	0	0
14569	3,4-$Cl_2C_6H_3$	H	191—193		11·6	$C_{19}H_{14}Cl_4N_2O_4S_2$		11·85	52	0	0	0
14572	3-$CF_3 \cdot C_6H_4$	H	164—165		11·6	$C_{21}H_{16}F_6N_2O_4S_2$		11·9	14	0	0	0
15290	MeCO	H	226	7·8	18·7	$C_{11}H_{14}N_2O_6S_2$	8·4	19·2	0	0	0	0
14530	Me	Me	116—117	9·4		$C_{11}H_{18}N_2O_4S_2$	9·15		100	0	0	0
14531	Et	Et	(c)	7·9		$C_{15}H_{26}N_2O_4S_2$	7·7		100	90	63	0
14567	pentamethylene		156	7·6	16·4	$C_{17}H_{26}N_2O_4S_2$	7·25	16·6	0	0	0	0
14536	$(CH_2)_2 \cdot O \cdot (CH_2)_2$		149—151		16·75	$C_{15}H_{22}N_2O_5S_2$		16·4	0	0	0	0
14570	Ph	Me	101—103		14·5	$C_{21}H_{22}N_2O_4S_2$		14·9	0	0	0	0
14678	$PhCH_2$	Me	104—106		14·0	$C_{23}H_{24}N_2O_4S_2$		14·0	25	0	0	0
15025	$CH_2 \cdot CN$	Me	111		17·8	$C_{13}H_{16}N_4O_4S_2$		18·0	86	23	0	0
15171	MeO	Me	99—100	8·3		$C_{11}H_{18}N_2O_6S_2$	8·3		0	0	0	0
15198	MeCO	Me	121—122		17·3	$C_{13}H_{18}N_2O_5S_2$		17·7	34	0	0	0
15199	p-$TolSO_2$	Me	140—142	4·8	20·8	$C_{23}H_{28}N_2O_6S_4$	4·8	21·8	0	0	0	0
14679	EtO·OC	Me	—		15·4	$C_{15}H_{22}N_2O_6S_2$		15·2	0	0	0	0
14815	EtO·OC	4-ClC_6H_4	178			$C_{25}H_{24}Cl_2N_2O_6S_2$			0	0	0	0
14816	α-NphO·OC	Me	170—172		10·1	$C_{31}H_{26}N_2O_6S_2$		10·4	0	0	0	0

(*a*) This product possibly still contains traces of dodecylamine hydrochloride.
(*b*) Lit.,[12] m.p. 187°—189°. (*c*) A viscous oil.

Table IV

N-RR'-substituted toluene-p-sulphonamides

R.D. No.	R	R'	M.p. °c	Reference	Found, % N	Found, % S	Formula	Required, % N	Required, % S	% Mortality to sheep blowfly larvae at concentration 0·25%	0·05%
14680	Me	H	78—79				$C_8H_{11}NO_2S$			0	0
14674	Ph	H	99—102				$C_{13}H_{13}NO_2S$			0	0
15200	MeCO	H	138—139	13			$C_9H_{11}NO_3S$			0	0
15288	p-$TolSO_2$	H	168—169				$C_{14}H_{15}NO_4S_2$			0	0
14106	Et	Et	55—56				$C_{11}H_{17}NO_2S$			65	0
14675	$PhCH_2$	Me	94—96				$C_{15}H_{17}NO_2S$			0	0
14676	$PhCH_2$	Ph	140—141	13			$C_{20}H_{19}NO_2S$			0	0
15021	$CH_2 \cdot CN$	Me	76—78		13·1	13·9	$C_{10}H_{12}N_2O_2S$	12·5	14·3	51	0
15020	$CH_2 \cdot CN$	Ph	113—115			11·0	$C_{15}H_{14}N_2O_2S$		11·2	0	0
15035	$CH_2 \cdot CN$	3,4-$Cl_2C_6H_3$	94—95			9·3	$C_{15}H_{12}Cl_2N_2O_2S$		9·0	0	0
15170	MeO	Me	60—62		6·5		$C_9H_{13}NO_3S$	6·5		16	0
15172	MeCO	Me	57—59	13			$C_{10}H_{13}NO_3S$			0	0
15173	p-$TolSO_2$	Me	124—126	14			$C_{15}H_{17}NO_4S_2$			0	0
14677	EtO·OC	Me	—			12·8	$C_{11}H_{15}NO_4S$		12·45	0	0
14736	EtO·OC	Ph	99—102	15		10·0	$C_{16}H_{17}NO_4S$		10·0	0	0
14737	α-NphO·OC	Me	111—113			9·1	$C_{19}H_{17}NO_4S$		9·0	0	0

Tol = toluene Nph = naphthalene

were prepared. The most active compound, toluene-2,4-disulphonamide, and the corresponding monosulphonamide were selected, and a wide range of *N*-substituents were introduced.

N-Aryl substituents brought about complete loss of activity, as did the larger alkyl groups. The fact that some activity was retained with smaller alkyl groups encouraged the preparation of derivatives bearing other small substituents such as cyanomethyl and methoxy. Acetyl and toluene-*p*-sulphonyl groups were also introduced. In every case, less active compounds resulted. Where the *N*-substituted disulphonamides showed some activity, the corresponding *N*-substituted monosulphonamides in Table IV were less active.

Where possible, attempts were made to combine the active disulphonamide structure with those of other insecticidal compounds. For example, the α-naphthoxycarbonyl derivatives in Table III and IV may also be regarded as arenesulphonyl derivatives of carbaryl (α-naphthyl *N*-methylcarbamate). Similarly, the 4-*p*-chlorobenzylthio derivative in Table I includes part of the structure of chlorbenside (*p*-chlorobenzyl *p*-chlorophenyl sulphide),[19] a highly persistent acaricide. These compounds were devoid of activity.

Among other compounds tested and found to be inactive were toluene-2,4-di(sulphonyl chloride), toluene-2,4-disulphonhydrazide and the dimethyl ester of toluene-2,4-di(sulphonic acid).

The activity of the disulphonamides was quite specific to blowfly larvae. They showed no insecticidal activity against the adult blowfly, some species of ticks and the adult and larval stage of the yellow fever mosquito (*Aedes aegypti*).

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Boots Pure Drug Co. Ltd.,
Chemistry Division,
Research Dept.,
Station Street,
Nottingham
and
Veterinary Science Division,
Thurgarton,
Notts.

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THE CYANOGENIC GLUCOSIDE CONTENT OF CASSAVA AND CASSAVA PRODUCTS

By T. WOOD

A simple and rapid procedure for the assay of the cyanogenic glucoside in cassava preparations has been developed. The HCN is liberated by autolysis of the ground tissue followed by distillation with acid into sodium carbonate solution, and estimated from the orange colour developed with picric acid.

The reproducibility and reliability of the assay was evaluated using plant material and aqueous extracts of the glucoside. Storage of plant tissue and extracts at −20° diminishes the rate of breakdown of the glucoside.

Values were obtained for the HCN content of peeled cassava tuber, cassava leaves, cassava peel, konkonte flour and garri. More than 100% variation was found between the content of neighbouring sectors of the same tuber. It was confirmed that the peel is a rich source of glucoside.

Introduction

Cassava, *Manihot utilissima*, or manioc as it is otherwise known, is widely used throughout Africa as a staple item of diet. It shares with the lima bean (*Phaseolus lunatus*) the distinction of being one of the few food crops containing dangerously toxic amounts of hydrocyanic acid after harvesting. Over the last 100 years or so, many investigators have analysed cassava with a view to understanding the factors which govern the amount of the cyanogenetic glucoside, linamarin, in the plant, but the evidence obtained has proved to be contradictory and confusing (see Jones[1] for a review).

A fuller understanding of this problem and of the function of the glucoside in the plant may conceivably be reached by studying the paths by which the glucoside is synthesised and broken down. The purpose of the present investigation was to develop a simple and sensitive assay method for the cyanogenetic glucoside in cassava, which could be applied to plant tissues, plant extracts, and chemical fractions of these, and to find a rich source of the glucoside for experimental purposes.

Experimental

Materials

Cassava tuber.—Tubers were from plants (variety Ankara) grown on the University Farm. They were transported directly to the laboratory after harvesting and samples taken for analysis the same day or within three to four days. After a few days, tubers began to go mouldy or otherwise deteriorate and were rejected. Transverse sections were cut from the tuber and the peel removed before analysis.

Cassava peel.—The outer bark was removed by scraping to reveal the purple outer surface of the peel. Two parallel cuts with a scalpel enabled the peel between the cuts to be removed easily in one piece by prising it up along the natural boundary between the peel and the remainder of the tuber. This piece was usually between 1 and 2 mm. thick.

Cassava leaves.—Fresh leaves were gathered and analysed immediately.

Garri.—Garri is a traditional preparation of peeled cassava which has been grated and lightly roasted after a spontaneous fermentation.

Konkonte flour.—Konkonte is a traditional product consisting of peeled cassava. Samples were obtained from the University Farm after they had been dried by roasting and ground to flour.

Aqueous extracts.—Extracts of the peel were made in ice-cold 0·025M–morpholine acetate buffer, pH 8·5. A suitable weight of tissue (4–12 g.) was taken, chopped finely with a sharp knife and ground with an equal weight of sand, firstly with sand alone and then after the addition of sufficient morpholine buffer to form a thick slurry. More buffer was added and the grinding repeated, then kieselguhr was added and the mixture filtered rapidly at the pump

through a thin layer of kieselguhr into a flask cooled in ice. The total volume of extractant and washings was about 3 ml./g. of tissue and the whole procedure took 15–20 min. The extracts were frozen immediately and stored at — 20°.

Frozen samples.—Samples of peel and peeled tuber weighing ~ 4 g. were wrapped tightly in ' Parafilm ' wax foil and stored at — 20°.

Standard cyanide solution.—Sodium cyanide was dried over conc. sulphuric acid and used to prepare a stock solution containing 181 mg. in 100 ml. A standard solution containing 0·100 mg. of hydrogen cyanide (HCN)/ml. was prepared by diluting 25 ml. of the stock solution to 250 ml. The stock solution could be stored for 1 to 2 months at 30° without deterioration.

Analytical methods

Assay of HCN.—Hydrogen cyanide was liberated from the plant material by autolysis followed by treatment with acid. It was distilled into sodium carbonate solution and later reacted with picric acid to yield orange-coloured isopurpuric acid.[2]

(*a*) *Distillation of HCN.*—Samples of plant material (1–4 g.) were weighed to the nearest 10 mg., finely chopped, and rapidly ground with sand and morpholine buffer. The resulting slurry was immediately transferred to a 250-ml. Claisen flask and the flask stoppered. The total volume of the contents was about 15 ml. The flask was incubated at 37° for 2 h. to permit autolysis to proceed. At the end of this time, the rubber stopper was replaced by one carrying two glass tubes arranged so that a stream of air could be drawn through the liquid in the flask and then led to a trap containing 2·0 ml. of 5% w/v sodium carbonate. To the contents of the flask were added a few drops of octyl alcohol and 15 ml. of 2N-sulphuric acid. The temperature of the distillation flask was raised to 100° by immersion in a boiling water-bath and was maintained at 100° for 30 min. with a steady stream of air passing through it. The trap containing the sodium carbonate was removed, the contents and washings were transferred to a 25 ml. graduated cylinder and made up to a convenient volume between 8·0 and 15·0 ml. A suitable aliquot was taken for the development of the colour with picric acid.

(*b*) *Development of colour.*—The following volumes were measured with a pipette into dry 1 × 15 cm. Pyrex test-tubes: 4·25 ml., or less, of the sample, 1·00 ml. of saturated picric acid solution (1·4% w/v at 30°), 1·00 ml. of 5% w/v sodium carbonate (or the difference between 1·00 ml. and the equivalent volume of 5% w/v sodium carbonate in the sample). The solutions were mixed and set aside for a few minutes to initiate colour development before addition of water to a final volume of 6·25 ml. The tubes were stoppered by corks wrapped with PVC film and the contents mixed. After being heated for 12 min. in a boiling water-bath, the tubes were cooled and the optical densities of the solutions read at 530 mμ in a spectrophotometer. Standards were run at the same time and were prepared from 0·10, 0·20, 0·50 and 1·00 ml. of the standard sodium cyanide solution. The HCN content was expressed in mg./kg. of fresh plant material.

Results

The assay procedure

The method of liberating HCN was based on that described by Seifert[3] except that the prewashing of the incoming air was found to be unnecessary and the HCN was absorbed in sodium carbonate solution instead of caustic soda. The development of colour was as described by Snell & Snell[4] except for the proportions of the reagents. A plot of the absorption curves of a standard and the blank over the range 480–550 mμ confirmed that 530 mμ was the most advantageous wavelength at which to measure the colour produced. Small variations in the amount of sodium carbonate in the reaction mixture had no appreciable effect on the colour intensity. Beer's Law was obeyed over the range 0–0·15 mg. of HCN per 6·25 ml. of solution. The slope of the standard curves varied within the limits ± 20% of the mean slope. Amounts of cyanide down to 0·02 mg. per sample could be measured.

Reliability of the assay

Standard amounts of sodium cyanide containing 0·1 mg. and 0·5 mg. were distilled in triplicate for 30 min. and values within ± 4% of the true value were obtained. In order to

obtain some idea of the length of time required to distil over the HCN from plant material, experiments were carried out on an extract of peel which was stored at — 20°. Aliquots of 2·00 ml. of this extract were assayed after various distillation times. The results (Table I) indicate that the greater part of the HCN is carried over in 30 min. and most of this distils over in the first 15 min.

Duplicate samples were prepared by quickly grinding 8·0 g. of peeled tuber with 8·0 g. of sand and weighing equal amounts of the mixture into two assay flasks. The assay values for these samples (Table II) were within ± 5% of their mean value. In general, replicate assays of extracts gave values within ± 10% of their mean.

Factors interfering with the assay

It has been reported[4] that aldehydes, acetone and hydrogen sulphide give a red colour with alkaline picrate. Since acetone is often used in the laboratory and may also be a normal constituent of plants, its effect was investigated. The same depth of colour was given by 500 mg. of acetone as was obtained with 1 mg. of HCN. It was concluded that traces present in plant material would be unlikely to interfere, but that there was a definite danger of false values being obtained if acetone was used to rinse apparatus employed in the assay. Since treatment of small (1–2 g.) amounts of *Canavalia ensiformis* and *Canavalia gladiata* gave no detectable increase in optical density of the sample over that of the blank, it was concluded that passage of air through the absorbing system did not result in the accumulation of detectable amounts of HCN derived from the atmosphere. Moreover, this demonstrated that treatment of plant material *per se*, at least in these cases, did not produce artefacts able to react with picric acid to yield an orange colour. The finding of very low HCN values for samples of stored garri (Table III) was additional evidence on this point.

Glucoside content of cassava tuber and peel

A number of neighbouring sectors (each 4 g.) were cut from a cross-section of the peeled tuber. Two of these sectors were assayed immediately, one was ground with sand and duplicate samples prepared as described above, and two were stored at — 20° and assayed 20 days later.

Table I

Assay of an extract of cassava peel

Aliquots of 2 ml. of the extract (containing approx. 0·4 mg. of HCN) were added to 15 ml. of water, incubated for 2 h. at 37°, then 15 ml. of 2N-H_2SO_4 added and the mixture distilled

Time of distillation, min.	Assay value, mg. HCN/kg.	Assay value as % of the mean of the 30 min. values
15	510	95
30	484	90
30	564	105
30	564	105
120	623	116

Table II

HCN content of neighbouring sectors of the peeled tuber and of the adjoining peel

Sector	Treatment	Assay value, mg. HCN/kg.
A	assayed immediately	81
B	assayed immediately	190
C*	duplicate after grinding with sand	102
	duplicate after grinding with sand	113
D	stored at — 20° for 20 days	45
E	stored at — 20° for 20 days	117
Peel	assayed immediately	705

Mean value for sectors of tuber = 108 ± S.D. 48 mg. HCN/kg.

* See text for further details

Table III

The HCN content of cassava and cassava products

Sample no.	Description	HCN (mg./kg. fresh wt.)	Sample no.	Description	HCN (mg./kg. fresh wt.)
A	Peeled cassava tuber	6	J	Cassava peel (by assay of aqueous extract)	577
B	Peeled cassava tuber	8	K	Cassava peel (by assay of aqueous extract)	538
C	Peeled cassava tuber	9	L	Cassava peel (by assay of aqueous extract)	280
D	Peeled cassava tuber	34	M	Cassava peel (by assay of aqueous extract)	224
E	Peeled cassava tuber	68	N	Cassava peel (by direct assay)	705
F	Peeled cassava tuber	80	O	Fresh garri	25
G	Peeled cassava tuber	94	P	Stored garri (sample O stored 1 month)	2
H	Fresh leaves (from same plant as G)	83	Q	Konkonte flour	20
I	Fresh leaves	162			

mean content of tuber (7 samples) = 43 ± S.D. 37 mg. HCN/kg.
mean content of peel (5 samples) = 465 ± S.D. 207 mg. HCN/kg.

A sample of the peel was also taken and assayed immediately. The results (Table II) indicate that there is more than 100% variation between the HCN content of neighbouring portions of the same tuber and, as expected from earlier reports,[5, 6] the content in the peel was several times higher than that of the interior. Storage at — 20° appeared to have preserved some, if not all, of the glucoside, but a definite decision on this would require the analysis of enough samples to allow statistical methods to be applied. Assay of an extract of peel and the residue after extraction showed that over 90% of the glucoside was extracted by the procedure used. Periodic assays of an extract showed that storage at — 20° partially preserved the glucoside, but that about half of the amount initially present had disappeared after 13 days (Fig. 1).

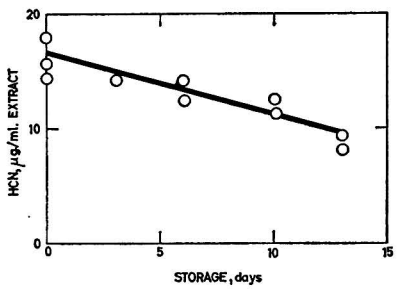

FIG. 1.—*HCN content of a cassava peel extract stored at —20°*

The HCN content of samples of peeled tuber covered the range 6–190 mg./kg. (mean 43, S.D. ± 37) and the content of the peel the range 224–705 mg./kg. (mean 465, S.D. ± 207) (Table III).

Glucoside content of other samples

The samples of garri and Konkonte flour, which are materials manufactured in a manner designed to reduce their content of poisonous glucoside, still contained appreciable amounts of cyanide, although this appeared to be lost from garri on storage.

Effect of autolysis

An idea of the rate of enzymic cleavage of the glucoside by endogenous linamarase was obtained by adjusting an extract of peel (containing the equivalent of 280 mg./kg. of HCN) to pH 5 with potassium hydrogen phthalate and incubating at 37°. At the end of 2 h. the extract was left exposed to the atmosphere at 30° for a further 2 h. to allow the liberated HCN to diffuse away. Subsequent assay showed that the HCN content had fallen to the equivalent of 56 mg./kg. A similar extract (containing the equivalent of 577 mg./kg. of HCN) after storage for 12 days at 0° contained only 26 mg./kg.

Discussion

The optimal pH for the action of linamarase has been reported to be similar to that of emulsin and in the range 5–6.[3] To diminish the breakdown of linamarin by endogenous linamarase during the grinding procedure, a buffer at pH 8·5 was used for extraction and good yields of the glucoside were obtained. The presence of active enzyme in the buffered extract and the breakdown of the glucoside at 0° and 37° was demonstrated. The rate of breakdown could be reduced considerably by storing the extract in the frozen state at — 20° and the precision of the assay was investigated using aliquots of such an extract.

The assay method which has been developed is simple and fairly rapid and proved to give reproducible results sufficiently precise for the purposes of comparing different samples and following changes in the HCN content of various preparations. The application of the assay to samples of tuber and peel was made considerably easier by storing the samples at — 20°, since a number of tubers could be harvested, brought to the laboratory, and sampled on the same day and assayed over the next one or two days.

The content of HCN in the peeled tuber of the small number of samples that were analysed covered the range 6–190 mg./kg., which may be compared with the range 30–370 mg./kg. reported by Greenstreet & Lambourne[5] and of 29–213 mg./kg. found by Oyenuga & Amazigo,[6] and the range 31–151 mg./kg. reported by Bolhuis[7] for the variety *Basiorao*. The mean value for the peel was over 10 times that of the mean value for the remainder of the tuber, a result similar to that of Oyenuga & Amazigo.[6] Consequently, the peel would seem to provide the most suitable tissue for a more detailed investigation of the metabolism of the glucoside.

It is generally recognised that tubers show an extremely wide variation in HCN content, even when selected from the same variety grown under the same conditions, and even when taken from the same plant.[1] However, it was surprising that this variation extended to neighbouring sectors of the same tuber and leads one to ask to what extent the reported variations between tubers have been influenced by the choice of the portion of the tuber to be examined. It may be that the most satisfactory method would be to homogenise the whole tuber before sampling. Since an active degradative enzyme is present and the time required for homogenisation would be considerable, the difficulties involved in this approach are obvious. Alternatively, a series of samples from different parts of the tuber could be assayed and the results treated statistically. However, since the object of the present investigation was primarily to find a rich source of the glucoside and a suitable method for assay rather than to obtain data for agricultural purposes, the problem was not pursued further.

Conclusions

The method of assay described gives good recoveries of HCN from standard amounts of sodium cyanide and appears to be reliable and suitable for the assay of the cyanogenic glucoside, both in cassava and in preparations derived from it. A complete evaluation of the method awaits the isolation of linamarin and the assay of known amounts by the above procedure. Storage of plant tissue and tissue extracts at — 20° for periods of a few days slowed the breakdown of linamarin and was a suitable means of preserving them for short periods. Extracts of cassava peel were rich in linamarin, and hence the peel would be a suitable tissue for further investigations of this compound and its metabolic relationships within the plant.

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Faculty of Pharmacy
Kwame Nkrumah University of Science & Technology
Kumasi, Ghana

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AVAILABLE LYSINE IN MEAT AND MEAT PRODUCTS

By Z. DVOŘÁK and I. VOGNAROVÁ

The amount of available lysine in different sorts of beef and pork was determined and the relation between lysine and content of connective tissue was ascertained. The extent of reduction of the available lysine on heat processing depends on the time of heating and the proportion of meat and meat products water lost. The presence of added sugars in meat is practically without influence. The amount of available lysine in meat can also be decreased by smoking and salting with nitrite. The values obtained for the different kinds of meat products are discussed.

Introduction

Animal proteins contain a relatively high amount of lysine, which is an important source of the amino-acid in human nutrition.[1] The proportion of lysine varies in different muscle proteins, substantially less being present in the connective tissue than in the myosin fraction.[2,3] The present study was undertaken to determine the dependence of the available lysine content on the composition of meat, including the proportion of connective tissue, the effect of spoilage, of heat processing and of reduction of water content.

Because of the free ε-amino-group in lysine, its availability may be decreased by processing. In this connexion, the most important factor is the effect of heat in presence of sugars.[4] The reaction of amino-groups with sugars is well known in dehydrated meat, where it can occur without heating to high temperature,[5] but less is known as to what extent these changes occur in non-dehydrated meat.

Further, the available lysine in meat is influenced by other reactions, e.g., the reaction of nitrite with the free amino-group of proteins causes the destruction of free ε-amino-group of lysine bound in proteins. This group may also be blocked by aldehydes, especially formaldehyde, which is always present in smoke. These reactions are also considered here.

Experimental

Samples of raw meat were taken from the slaughterhouse, and from each kind seven samples were analysed irrespective of the sex and age of the animals. The samples were chosen so that the particular parts contain predominantly the following muscles: beef fillet and pork loin: *psoas major*; beef and pork shoulder: *triceps brachii* and *latissimus dorsi*; beef loin: *longissimus dorsi* and *quadratus lumborum*; beef and pork round: *biceps femoris, glutaebiceps*; beef flank: *transversus abdominis, rectus abdominis*; ribs: mostly *intercostales interni*; beef neck: *sternocleidomastoideus, trapezius*; pork spare rib (neck): *semispinalis capitis, complexus major*; beef shank and pork feet: all muscles.

All samples were well ground and then homogenised with water in a ratio of 1 weight part of meat to 4 vol. parts of distilled water. This homogenate was used for the analyses. Nitrogen and dry matter were determined by conventional methods. Carpenter's method with fluorodinitrobenzene was used for the determining of available lysine.[6] Tryptophan was determined according to Roth & Schuster[7] and hydroxyproline according to the method of Neumann & Logan.[8]

To study the onset of deterioration of meat, samples of beef fillet were hung in a refrigeration box at a temperature of + 10°. Ammonia as a criterion of deterioration was determined according to Conway.[9]

The experiments dealing with effect of smoke were carried out thus. The smoke was generated in a smoke generator from beech sawdust heated to 320° and tarry compounds were condensed out. The smoke was passed with air at a temperature of 20° at a rate of 5 m.³/min. through a suspension of the meat.

Net protein utilisation (NPU) was determined according to the procedure of Miller & Bender.[10]

Examination of the effects of formaldehyde and nitrite was made with beef fillet homogenised (1 weight part of meat) with water (10 vol. parts), which was kept with the additives for 48 h. at + 20°.

Results

(1) *Dependence of available lysine on the composition of meat*

(a) *In beef and pork.*—In Table I are shown average values of available lysine in different cuts of beef and pork. The difference between the extreme values is about 3 g. of lysine per

Table I

Available lysine in raw beef and pork

(Average for seven samples: values ± represent standard deviations)

Cut of beef	Available lysine, g./16 g. N	Cut of pork	Available lysine, g./16 g. N
Fillet	9·15 ± 0·48	Shoulder	8·94 ± 0·54
Round	8·72 ± 0·75	Loin	8·59 ± 0·10
Loin	8·00 ± 0·42	Spare rib (neck)	8·14 ± 0·58
Shoulder	7·90 ± 0·34	Round	7·94 ± 0·37
Ribs	6·82 ± 0·49	Ribs	7·11 ± 0·70
Neck	6·80 ± 0·50	Hind feet	5·66 ± 0·18
Flank*	6·64		
Shank (hind)	6·53 ± 0·32		

* Value obtained from the relation of connective tissue (content of available lysine 3·02%) to whole meat (content of available lysine 7·85%), which is calculated as 1 : 3

100 g. of protein, probably due to unequal distribution of the connective tissue proteins. For example, myosin or tropomyosin contain more than 10% of lysine (11·92% and 15·70% respectively),[2] but the proteins of connective tissue contain less lysine. Collagen from beef Achilles tendon contains 4·9% and elastin from *ligamentum nuchae* 0·40% of lysine.[3] The results obtained show the dependence of available lysin on the protein content of connective tissue and of other proteins present in meat.

(b) *Relation between the content of available lysine and the amount of connective tissue.*—The ratio of tryptophan to hydroxyproline may be a criterion for the amount of connective tissue in meat.[11] This criterion is based on the fact that in connective tissue proteins tryptophan is absent, but hydroxyproline is present in a great amount, whereas in other proteins of the non-collagen type this situation is reversed. In Fig. 1 is shown the relation between the tryptophan/hydroxyproline ratio to the available lysine content in various beef cuts. Over a small range of values it can be assumed that the relation is linear, so that it may be concluded that the amount of available lysine in raw meat is in indirect proportion to the content of connective tissue.

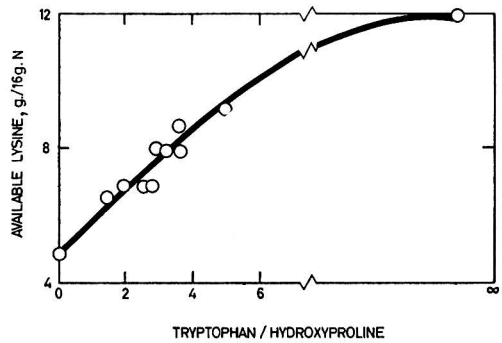


FIG. 1.—*Relation between available lysine and the relation tryptophan/hydroxyproline in beef*
(Point on the ordinate represents value for acid-soluble collagen,[8] the other extreme point represents value for myosin[2])

(c) *Relation between the content of available lysine and spoilage of meat.*—Some samples of spoiling beef fillet were examined with the results shown in Fig. 2. The amount of ammonia, determined by the microdiffusion method, is taken as a criterion for oxidative spoilage of meat.[12] From Fig. 2 it is evident that the results obtained from such samples are very variable with a tendency to reduce the content of available lysine, but not in proportion to the degree of spoilage as determined by ammonia content. Decreased lysine values are probably due to different metabolism of the amino-acid by bacteria which influence the deterioration of meat.

(2) *Influence of heat on reduction of the content of available lysine in presence of sugars*

Samples of beef fillet were ground and 2% of sodium chloride followed by 2% of glucose or 2% of potato starch was added. The meat was packed in cans (content 200 g.) and heated for different times in an autoclave as shown in Table II. The values for lysine content are expressed on the raw meat parts, in which the content of available lysine is taken as 100.

From the results shown it is seen that the amount of available lysine decreased at increased temperatures and longer times. There is no significant effect of the added glucose and starch. An attempt was made to break down the glucose present in the meat by treatment with glucose oxidase with catalase at 20° for 3 days, but after this glucose would still be detected qualitatively. It was not possible therefore to verify if a Maillard reaction occurs in muscle.

(3) *Influence of decreased content of water in meat and meat products on the availability of lysine*

The incidence of the Maillard reaction is observed particularly in dehydrated food. For this reason the content of available lysine was studied in beef fillet dehydrated by freeze drying to a water content of 1·7% and also in dehydrated meat products. The results in Table III

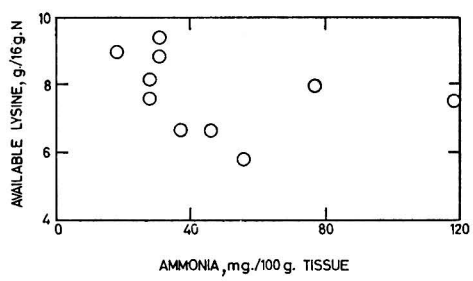


FIG. 2.—*Relation between available lysine and spoilage of beef*

Table II

Decrease (%) of available lysine in beef fillet in relation to heat treatment, time and addition of sugars
(raw fillet = 100)

Time of heat treatment, h.	70°		121°			140°			160°		
	Without addition	With glucose	Without addition	With glucose	With starch	Without addition	With glucose	With starch	Without addition	With glucose	With starch
1		94·0	86·8	88·1		64·8	69·3		64·8	59·3	68·8
2			81·0	75·0	73·9	67·2	65·0				
3	92·0	87·6	78·5	70·5		57·8	60·3		50·3		52·8
4			65·3	64·5	56·5		49·8	53·1			
4½	77·3	70·8									

show a striking decrease of available lysine in freeze-dried raw meat. Subsequent storage for one year caused a further decrease of 40·7% in lysine available. It is evident that decrease of water alone is insufficient for the reduction in available lycine and that time is of great importance. Similar influences, but complicated by processes such as smoking, heatprocessing or addition of various ingredients can be observed in dried salamis. This is evident especially in Hungarian salami and Tourist salami. Although in these products the content of available lysine is already low at time of their sale, further decrease is evident after storage for one year. The salamis in Table III were dried for some weeks or months. The Common dry salami and Highlander salami were dried for a shorter time, so that in the former there was only a small decrease of available lysine. The greater decrease in Highlander salami is due (similarly also in Hungarian salami and Gyulai salami) to protracted influence of cold smoke (see below).

From the results it follows that the availability of lysine decreases with the grade of dehydration of meat, but the decrease is dependent on the time during which the reaction takes place.

(4) *Influence of smoking on the content of available lysine*

(a) *Influence of smoke.*—A striking decrease of available lysine was found in meat products when they were exposed to cold smoke for 1–2 days. Typical examples are Tea sausages, made only from pork and smoked by the given manner. Analysis of samples of the same charge before and after smoking gave values 7·77 and 6·83 g. of lysine per 16 g. of N respectively, so that the decrease is 12·2%.

In a further experiment the suspension of meat proteins was bubbled with smoke at a temperature of + 20°. As smoke may contain nitrogenous compounds, the results were related to the amount of nitrogen before smoking. The results in Fig. 3 show that the amount of available lysine decreases proportionately with the amount of bubbled smoke.

(b) *Effect of formaldehyde.*—The effective constituents of smoke are phenolic and carbonyl compounds, the most effective of the latter being formaldehyde, which was found[13] to be present

Table III

Available lysine in meat and meat products with reduced water content
(Average for seven samples: values ± represent standard deviations)

Meat or meat product	Water content, %	Available lysine, g./16 g.N	Average lysine calc., g./16 g.N	Lysine present as % of average lysine	Material used and manner of processing
Beef fillet					
7 days after freeze drying	1·7	9·48			
1 year after freeze drying (stored in air at + 20°)	4·8	5·62	9·48	59·3	
Hungarian salami original as sold	26·0	5·33 ± 0·19	8·49	62·9	Pork; smoked 2 days with cold smoke
Sold after 1 year storage at + 20°	15·0	4·77 ± 0·17	8·49	56·3	Pork; smoked 2 days with cold smoke
Tourist dry salami original as sold	39·0	5·92 ± 0·22	8·27	71·5	Pork and beef; smoked 2–3 h. at 70°
Sold after 1 year storage at + 20°	20·0	5·36 ± 0·27	8·27	64·9	Pork and beef; smoked 2–3 h. at 70°
Gyulai salami	25·0	5·48 ± 0·11	8·49	64·6	Pork; smoked 2 days with cold smoke
Common dry salami	50·0	6·02 ± 0·22	6·41	93·9	Beef and pork; smoked 1–2 h. at 70°
Highlander salami	52·0	5·86 ± 0·21	8·37	70·0	Pork and beef; after heat processing (70°), smoked 1 day with warm smoke

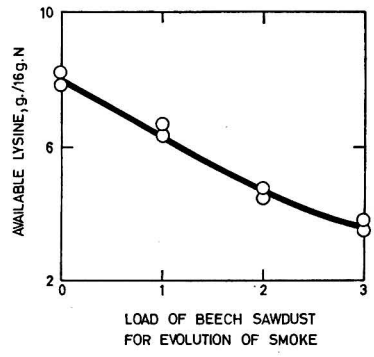


FIG. 3.—*Effect of smoke on available lysine in filtrate from meat extract*
(Meat extract prepared by homogenisation of 1 part of beef with 4 parts of water)

in the smoke from beech sawdust in concentration of 1·094 mg. in 1 m^3. Acetaldehyde also makes up a substantial part of the carbonyl compounds. The dependence of availability of lysine on log (concentration of formaldehyde) is linear as shown in Fig. 4. One can suppose that formaldehyde is the compound in smoke which lowers at low concentrations the amount of available lysine.

The lysine free-amino group is fixed in protein by formaldehyde. The loss of the availability is confirmed by the determination of NPU in young rats. In Table IV the NPU of raw beef

Table IV

Net protein utilisation in beef fillet and in formaldehyde-treated beef fillet

Protein tested	Nitrogen in rats of all the group, g.	Nitrogen in diet consumed, g.	NPU
Control group	3·17	0·18	
Beef fillet	5·73	3·74	73
Beef fillet after formaldehyde treatment	4·54	2·03	58

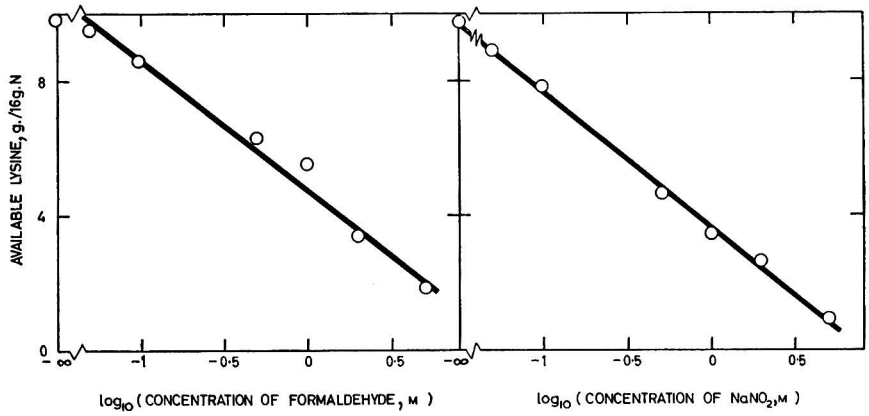
fillet is compared with that of the formaldehyde-protein precipitated from the beef fillet homogenate. The decrease of NPU may be explained mainly because of the loss of the availability of lysine.

(5) *Effect of nitrite on the amount of available lysine in meat*

Nitrous acid reacts with primary amines and breaks them down. Nitrites are used in the salting of meat to obtain a desirable colour, the usual amount being 0·5% of the sodium chloride used. Although nitrite ions in meat quickly reduce to nitrogen oxide, which forms the desirable colour with myoglobin, a study was made to see if nitrite ions in acidified meat react with the ε-amino group of lysine and destroy it. The results given in Fig. 5 indicate that this reaction does occur, the dependence of decrease in the amount of available lysine to log (concentration of nitrite) being similar to that with formaldehyde.

(6) *The amount of available lysine in meat products*

(a) *Meat products.*—According to the meat used in meat processing it is possible to ascertain what amount of lysine should be present in the final product. On this basis, in Table V the actual amount of available lysine in the samples is compared with the value obtained by calculation. It is evident that in common types of sausages the content of available lysine is reduced approximately by 20%. Decrease due to heat processing is seen from the values of



(FIG. 4, *left*).—*Effect of formaldehyde,* (FIG. 5, *right*) *of nitrite, on available lysine in beef homogenate*

Gipsy pork loins and Prague ham (calculated loss is 3·7% and 8·2% respectively). Greater reduction was observed in Tea sausages, due to the prolonged time of smoking (see above). Higher values than those calculated were found in canned meats and in Frankfurters. An increase of 29% is striking, especially in beef in natural juice.

(b) *Gelatination of collagen in meat products.*—Samples for the determination of available lysine were taken from cans in such a manner that for homogenisation only meat was used, without the juice developed during sterilisation. This juice is derived from collagen, which is relatively poor in available lysine, and this during sterilisation changes into gelatin and which dissolves in water present. Owing to this loss of collagen, the piece of meat is relatively enriched in available lysine content. This fact is confirmed by results given in Table VI. The determination of available lysine in the whole can of beef showed that the value found corresponded closely with the theoretical value (94·1%).

Table V

Available lysine in meat products

(Average for seven samples: values ± represent standard deviations)

Meat product	Available lysine, g./16 g.N	Lysine calc., g./16 g.N	Lysine present as % of average lysine	Material used and manner of processing
Dietetic frankfurters	7·01 ± 0·59	7·83	89·5	Pork and beef; 7–12 min. at 72–75°
Garlic sausages	5·21 ± 0·20	6·43	81·0	Beef and pork, meat from beef and veal heads, beef lungs, cooked pork skins; 15–25 min. at 75–78°
Parisian sausages	6·55 ± 0·12	7·30	89·8	Pork and beef, raw pork fat; 2–3 h. at 70–75°
Ham sausages	6·80 ± 0·11	8·27	82·3	Pork and beef; 1·5 h. at 70–75°
Cabanos	7·25 ± 0·45	7·22	100·2	Pork and beef; 20 min. at 75–78°
Tea sausages	6·25 ± 0·75	7·77	80·5	Pork; 48 h. in cold smoke
Meat loaves	6·35 ± 0·65	6·25	101·6	Beef and pork; 2–3 h. at 80–90°, then roasted
Gipsy pork loins	8·26 ± 0·70	8·59	96·3	Pork loin; 2 h. at 80°
Pork luncheon meat	7·71 ± 0·36	7·54	102·4	Pork, meat from pork heads; 60 min. at 121°
Pork in natural juice	9·48 ± 0·19	8·48	111·8	Pork, cooked pork skins; 70–90 min. at 121°
Beef in natural juice	9·81 ± 0·19	7·60	129·0	Beef; 60–100 min. at 121°
Frankfurter sausages	9·46 ± 0·18	8·40	112·6	Pork and beef; 30 min. at 104°
Prague ham	8·00 ± 0·23	8·72	91·8	Pork round; 2·5–3·5 h. at 75°

Table VI

Distribution of available lysine in canned beef shank

Sample	Available lysine, g./16 g. N	% distribution
Can with raw meat	7·45	100
The whole content of sterilised can	7·01	94·1
Meat part of can	8·70	116·7
Gelatin part of can	6·22	83·4

Discussion

The available lysine in food is easily lost, due in most foods studied to a Maillard reaction, especially in those processed in the dry state. Less attention has been given to natural foods where there is no reason to suppose such a loss. Meat and meat products are an exception because of the diversity of proteins present in them, and also because during processing, reactions occur which decrease the availability of lysine. This is especially true of the use of nitrite and of smoking.

Beside other essential amino-acids lysine is so important that its amount in plasma proteins is taken as a criterion of the biological value of food.[14] The results given for raw meat confirm this assumption. The amount of available lysine decreases with increasing amount of connective tissue. In the cuts of raw meat used the difference of available lysine in beef is 36%, in pork 43%. The dependence of the relation tryptophan/hydroxyproline and the amount of available lysine shows a direct relation, so that the amount of available lysine in raw meat may be determined indirectly if the relation tryptophan/hydroxyproline is known, or better if the proportion of connective tissue is known.

The availability of lysine in raw meat may be partly influenced by spoilage. The amount of ammonia in meat is taken as a measure of oxidative deterioration, a value greater than 30 mg./100 g. of tissue indicating the onset of spoilage. Later, the increase in ammonia is very rapid, an amount of 40–60 mg./100 g. of tissue signifying advanced spoilage.[12] It is interesting that the availability of lysine decreases at this stage—perhaps due to the metabolism of the protein-lysine by bacteria. But these results are not significant since in more advanced deterioration higher values of available lysine are found again.

As to the reaction of lysine with sugars, it can be said that in most meat products the decrease does not exceed more than 20% and additional glucose or starch has little effect, probably because there is enough glucose and glycogen in the muscle tissue for the reaction to occur. The experiments for removing all glucose from meat by treatment with glucose oxidase were unsuccessful. It is difficult to determine therefore if decrease of available lysine in meat on heat processing is due to a Maillard reaction, although it is probable. Lysine begins to decompose at 240°, so that under the conditions used here, direct thermal breakdown is unlikely.[15]

The effect of a Maillard reaction was quite striking in meat and meat products with reduced water content and the effect is enhanced by long storage. Dry salamis have lower available lysine content, the lower is their water content, the longer the dry product is stored. It must be added that the decrease in the availability of lysine in some of these products is due to smoking.

It seems that short-time smoking has no effect on the loss of availability, but such loss is evident when the smoking process proceeds for several hours or days. From the results it is apparent that the effective compound in the smoke is formaldehyde, whose effect on free amino-groups is well known. The bonding of formaldehyde with the ε-amino group of lysine is as firm as that with sugars. The Net Protein Utilisation of the proteins from meat where these groups were blocked with formaldehyde is decreased, indicating that the ε-amino group of lysine is not freed in the digestive tract.

A different kind of reaction is that of muscle proteins with nitrite. In the acid medium of meat it can occur that, before destruction of nitrite to nitrogen oxide and the development of colour, some of the nitrite ions react with free amino-groups in the meat proteins. As only a small amount of nitrite is added in meat products, this reaction is probably not significant as regards the availability of lysine.

A survey of several types of meat products is given in respect to their content of available lysine. If one compares the values found by determination with those calculated, some decrease of availability is seen. This decrease may be due to the influence of heat processing or to a Maillard reaction, and also to the effect of such factors as smoking and nitrite. The decrease here does not exceed 20%. Values higher than the theoretically calculated are found in most canned products. These may be explained by a relative enrichment of meat of lysine through a loss of gelatin.

If the content of lysine in meat is compared with that of other protein food, it is seen that meat and meat products are in every case one of the most important sources of lysine in food.[1] Nevertheless, in view of the difference in lysine values found in different meat cuts, and owing to different technological processes, the biological value of meat and meat products (as determined, e.g., by the index of essential amino-acids) may vary.

Meat Research Institute
Brno 12, Palackého 1–3
Czechoslovakia

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CHLOROPHYLL BLEACHING BY LEGUME SEEDS

By MARGARET HOLDEN

Legume seeds and extracts made from them bleached chlorophyll in the presence of long-chain fatty acids. The most effective acids were substrates for lipoxidase, but oleic acid and even palmitic and stearic acid were also active. The bleaching was inhibited by commercial antioxidants.

Chlorophyll was bleached by extracts which had lipoxidase activity but not by purified lipoxidase preparations. Addition of lipoxidase to seed extracts stimulated bleaching 2- to 3-fold with linoleic acid but only slightly with oleic acid.

The optimum pH for bleaching was about 6, and for linoleate hydroperoxide formation was near 8. Evidence is given that at pH 6 hydroperoxide was being destroyed.

Chlorophyll appears to be bleached by co-oxidation during a chain reaction involving peroxidation of fatty acid and the breakdown of hydroperoxide by a heat-labile factor.

Introduction

Strain[1] observed that chlorophylls *a* and *b* were oxidised to colourless substances in a system consisting of an aqueous extract from soya-beans, a fat and oxygen. Mapson & Moustafa[2] found that enzyme preparations from peas catalysed the oxidation of various substances, including chlorophylls, when linoleic (or linolenic) acid was added. The oxidation of glutathione in this system appeared to be the result of two reactions—one was due to the action of lipoxidase and was cyanide-insensitive, the other was cyanide-sensitive and possibly involved a metal catalyst. Wagenknecht *et al.*[3] found that chlorophyll was destroyed in frozen green peas which had not been previously blanched. They later obtained evidence[4–6] that this was mainly by lipoxidase action. The degradation of chlorophyll in frozen green beans was also related to peroxidation of fat by Walker.[7]

The oxidation of carotene by a lipoxidase–linoleate system has been studied by several workers.[8–10] Tappel[11] investigated the effect of various antioxidants on the co-oxidation of vitamin A and carotene during the haematin-catalysed oxidation of emulsified oleic acid. Blain & Styles[12] described a lipoperoxidase in extracts from soya-beans which bleached carotene during destruction of preformed peroxide, rather than during coupled concurrent oxidation of linoleate by lipoxidase action. Blain & Barr[13] considered that soya extracts contained too little haematin to account for the amount of hydroperoxide destroyed, and that if an iron porphyrin compound was the factor responsible, then its activity must be much greater than that of catalase, cytochrome *c* or peroxidase.

Dillard *et al.*[14] and Gini & Koch[15] also investigated a lipohydroperoxide breakdown factor in soya extracts. Koch & Gini[16] found that the factor was present in extracts of several legume seeds including navy bean, lentil and small red bean.

The present work on the enzymic ' bleaching ' of chlorophyll by legume seeds is part of a study of chlorophyll degradation in plant tissues during senescence and when they are attacked by pathogens.

Experimental

Materials

For many of the preliminary experiments, an 80% aqueous acetone extract of bean leaves (*Phaseolus vulgaris*) was used without separation of the chlorophylls from carotenoids. For other experiments, the chlorophylls were separated from xanthophylls and carotenes by chromatography on a column of polyethylene powder (Experimental resin QX 2187) kindly provided by the Dow Chemical Co. (U.K.) Ltd. Chlorophylls *a* and *b* were prepared by chromatographing the mixed pigments eluted from the polyethylene on a column of Whatman crystalline cellulose powder (CC31 or CC41).

Pheophytins, pheophorbides and chlorophyllides were made as previously described.[17]

Linoleic acid (96–97%), linolenic acid (99%), arachidonic acid (60%), oleic acid (97%), stearic acid (99%), ricinoleic acid (98%), α-elaeostearic acid and elaidic acid were obtained from L. Light & Co. Ltd., Colnbrook, Bucks, and trilinolein from the Hormel Foundation, Austin, Minnesota. To minimise autoxidation the acids were stored at 0° and ethanolic solutions were also kept cold.

Soya flour (DO-SOY) and one sample of soya-beans were a gift from the British Arkady Co. Ltd., Manchester. Soya-beans were also obtained from Heath & Heather, St. Albans, and from Thompson & Morgan (Ipswich) Ltd. Other samples of soya-beans and seeds of tropical legumes were obtained from Patak (Spices) Ltd., 134 Drummond Street, London, and R. A. Hustler Ltd., 55 Rupert Street, London, W.1. Named varieties of pea and bean seeds were bought from local seed merchants.

Enzymes.—Lipoxidase preparations were bought from L. Light & Co. Ltd. and from Seravac Laboratories (Pty) Ltd., Moneyrow Green, Holyport, Maidenhead, Berks.

Catalase was a crystalline preparation from bovine liver obtained from Sigma Chemical Co. Peroxidase was made from horseradish roots and kindly provided by Dr. P. J. G. Mann.

Antioxidants.—Progallin P (n-propyl ester of trihydroxybenzoic acid) was given by Nipa Laboratories Ltd., Treforest Industrial Estate. Santoquin (6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline) was given by Monsanto Chemicals Ltd., 10–18 Victoria Street, London. Nordihydroguaiaretic acid (NDGA) and butylated hydroxytoluene (BHT) were obtained from L. Light & Co. Ltd.

Methods

Preparation of extracts.—Seeds were soaked overnight in distilled water and the testas were discarded, and the cotyledons were ground with sand in a mortar in sodium acetate–acetic acid buffer solution, 0·2 M with respect to total acetate, pH 5·9 (3 ml./g. wet wt.). Extracts made by grinding in a high-speed macerator had considerably less bleaching activity than those made

in a mortar. The suspension was centrifuged at 5000 *g* for 10 min. and the deposit discarded. The same buffer was used to make extracts from soya flour and from pea powder that had been made by grinding dried peas in a hammer-mill.

Determination of chlorophyll bleaching activity

(a) *In seeds, seedlings, leaves, roots and flour*

A weighed amount of fresh material, of known dry-matter content, was ground rapidly with sand in a mortar in 14 ml. of acetate buffer solution pH 5·9, 0·1 M. A solution (4 ml.) of chlorophyll, about 0·5 g./l. in 80% v/v aqueous acetone, was added and the suspension whisked vigorously for 2 min. at room temperature (16–20°) in dim light. Acetone (52 ml.) was added to stop the reaction and dilute the pigments, and the suspension was then filtered. The absorption (A) of the filtrate was measured in an EEL photoelectric colorimeter with filter 607 and compared with the absorption (A_0) of untreated chlorophyll (4 ml. diluted to 70 ml. with buffer solution and acetone). $100(A_0 - A)/A_0$ is called % bleaching. When linoleic acid, or other long-chain fatty acid, was included in the system, 1 ml. of ethanolic solution 10 g./l. was added.

(b) *In extracts and enzyme preparations*

Enzyme solution (1 ml.) was added to 49 ml. of a mixture containing sodium acetate buffer pH 5·9, an acetone solution of chlorophyll and a fatty acid. The final concentration of acetate was 0·01 M, of acetone 12% v/v, chlorophyll about 0·08 g./1., fatty acid 0·2 g./l. and of ethanol 2% v/v. The mixture was shaken vigorously in a glass-stoppered flask covered with aluminium foil, and 5-ml. samples were removed at 1-min. intervals and added rapidly to 10 ml. of acetone. The acetone solutions were filtered and the absorption of the filtrate was measured with filter 607.

Determination of lipoxidase activity

(*a*) The formation of linoleate hydroperoxide was followed by the ferric thiocyanate method as described by Koch *et al.*,[18] except that high-grade absolute ethanol had to be used for stopping the action of the enzyme and diluting the reaction mixture, because 95% ethanol gave high blank values. The absorption was measured with filter 623.

(*b*) A method based on that of Theorell *et al.*[19] was used. The increase in absorption at 234 mμ, due to formation of conjugated diene hydroperoxides, was measured.

Absorption curves in the visible and ultra-violet regions were obtained with a Unicam SP 500 or with an Optica Recording Spectrophotometer.

Results

Preliminary experiments with soaked soya-beans ground in buffer solutions with pH values between 4·5 and 9·0 indicated that some chlorophyll was bleached over much of the range, with an optimum near pH 6 (Fig. 1). Bleaching was not observed with soya-beans that had been dropped in boiling water and kept there for 5 min. The bleaching was not dependent on light; it took place to the same extent in the dark as in dim daylight. The amount of bleaching at pH 6 varied with the number of beans used, and with the length of time of whisking with chlorophyll.

Additions of various long-chain saturated and unsaturated fatty acids greatly increased the bleaching (Table I) but several other acids had no effect. Trilinolein was less effective than linoleic acid. Boiled beans did not bleach chlorophyll in the presence of fatty acids. Fig. 2 shows the effect of adding different amounts of linoleic, oleic or stearic acid to the system. The pH optimum for bleaching in the presence of fatty acid was at about 6·3 (Fig. 3) and the activity dropped sharply above this value.

The presence of acetone in concentrations up to 25% by volume did not affect the bleaching, although greater concentrations were inhibitory. Bleaching was much greater, both with and without linoleic acid, in samples that were aerated by whisking vigorously with the pestle than those not aerated.

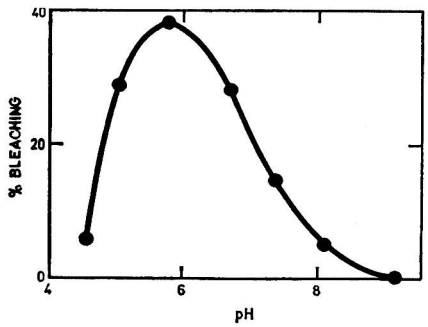

FIG. 1.—*Effect of pH on chlorophyll bleaching by soya-beans*

For each pH value 4 soaked soya-beans were ground for 3 min. with buffer and chlorophyll solutions as described under Methods, except that Michaelis veronal–acetate buffers were used for the whole pH range. The actual pH of the suspension was measured on a duplicate sample.

Of fifteen other legume seeds tested for chlorophyll-bleaching activity at pH 6 with and without added linoleic acid, black gram was the only one other than soya that caused appreciable bleaching without adding fatty acid. In Table II the amount of bleaching by various seeds with linoleic acid present is compared with that of a named variety of soya. All the seeds had bleaching activity and several species were nearly as active as soya; calvary clover had more activity than soya and French and broad beans and groundnuts all had only a little activity.

Table I

Effect of added fatty acids etc. on chlorophyll bleaching by soya-beans

(One soya bean was ground for 2 min. with acetate buffer solution pH 5·9, chlorophyll and acid as described under Methods: long-chain fatty acids added in ethanolic solution, the others in aqueous solution)

Additive	Bleaching, %	Additive	Bleaching, %	Additive,	Bleaching, %
No addition	8	Elaidic acid	60	Succinic acid	<5
Linoleic acid	69	Palmitic acid	45	Acrylic acid	<5
Linolenic acid	70	Stearic acid	46	Malic acid	<5
Elaeostearic acid	44	Ricinoleic acid	46	Maleic acid	<5
Arachidonic acid	62	Glycollic acid	9	Citric acid	<5
Oleic acid	63	Propionic acid	<5	Cinnamic acid	<5
				Trilinolein	21

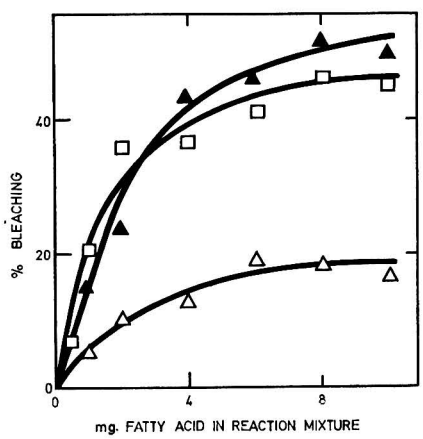

FIG. 2 (left).—*Effect of adding different amounts of fatty acids on chlorophyll bleaching by soya-beans*

(For each point 1 soya-bean cotyledon was ground with acetate buffer, chlorophyll and fatty acid solutions as described under Methods)

▲ oleic acid □ linoleic acid
△ stearic acid

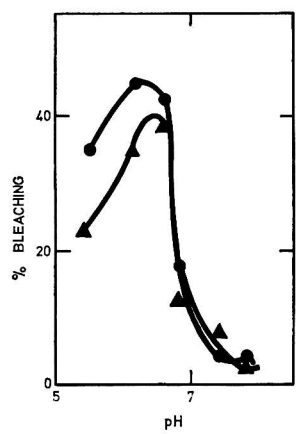

FIG. 3 (right).—*Effect of pH on chlorophyll bleaching by soya beans in the presence of linoleic and oleic acids*

(For each pH value 1 soya-bean cotyledon was ground with Michaelis veronal-acetate buffer, chlorophyll and fatty acid solutions as described under Methods)

● linoleic acid ▲ oleic acid

Table II

Chlorophyll-bleaching activity of legume seeds in the presence of linoleic acid

Seed	Variety	No. of seeds used	Weight before soaking, g.	Bleaching, %	Relative bleaching activity
Soya-bean (*Glycine max*)	Thompson & Morgan's 'Panda'	¼	0·04	43	100
	'Rothamsted'*	¼	0·05	47	94
Green gram (*Phaseolus radiatus* L.)		2	0·08	26	29
Black gram (*Phaseolus mungo* L.)		2	0·09	42	42
French bean (*Phaseolus vulgaris*)	Bee's 'Masterpiece'	1	0·44	36	7
Runner bean (*Phaseolus multiflorus*)	Cuthbert's 'Prizewinner'	½	0·72	49	6
Lupin (*Lupinus albus*)		½	0·20	44	20
Black-eye bean (*Vigna sinensis*)		½	0·16	43	25
Hyacinth bean (*Dolichos lab-lab*)		½	0·08	53	60
Calvary clover (*Medicago echinus*)		1	0·025	43	156
Pea (*Pisum sativum*)	'Giant Stride'	½	0·16	46	26
Groundnut (*Arachis hypogaea*)		1	0·42	15	3
Asparagus pea (*Tetragonolobus purpureus*)		2	0·09	53	53
White gram (*Cicer arietinum* var.)		¼	0·13	55	39
Bengal gram (*Cicer arietinum* var.)		½	0·08	55	62
Laburnum (*Laburnum* X *Vossii*)		2	0·08	44	50
Broad bean (*Vicia faba*)	Dobie's 'Dreadnought'	½	0·85	15	1·5

* Fresh seed from plants grown from beans from Patak (Spices) Ltd.

These results were obtained with samples of seeds with a germination rate greater than 90%, but seeds that were no longer viable also bleached chlorophyll.

Effect of germination on bleaching activity

Ungerminated pea seeds bleached chlorophyll only slightly unless a long-chain fatty acid was added, but on germination the cotyledons bleached strongly without added acid. Fig. 4 shows this in an experiment with Kelvedon Wonder peas with linoleic acid. Ten days after having been put to soak, the same amount of bleaching occurred whether or not linoleic acid was added.

Extracts from shoots and roots of young seedlings also had bleaching activity which disappeared, except from the cotyledons, from seedlings about 2 weeks old.

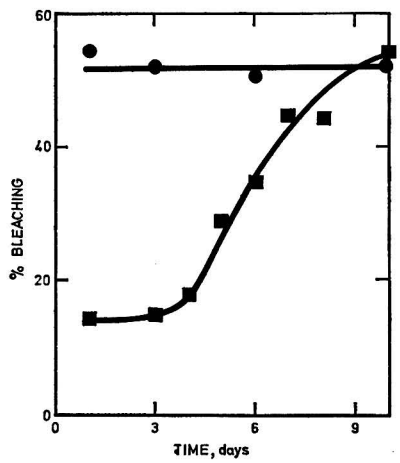

FIG. 4.—*Effect of germination on chlorophyll bleaching by pea seeds*

[At each sampling 2 peas, var. Kelvedon Wonder, were ground with acetate buffer and chlorophyll solutions as described under Methods. To one series 1 ml. of an ethanolic solution of linoleic acid (10 g./l.) was added and to the other 1 ml. of ethanol]

■ no linoleic acid ● 10 mg. linoleic acid

Effect of antioxidants

Siddiqi & Tappel[20] who studied the effect of various antioxidants on the activity of lipoxidases from several species of legume found that nordihydroguaiaretic acid (NDGA) was the most effective of the compounds tested. Blain & Shearer[21] found that NDGA prevented carotene bleaching in a system with methyl linoleate and soya flour extract. It was, however, less effective than butylated hydroxyanisole (BHT) when the carotene bleaching was caused by haemoglobin instead of

soya extract. Table III shows the effect of four commercial antioxidants on chlorophyll bleaching by pea, soya and white gram seeds with linoleic acid present. All the compounds considerably diminished the bleaching, Progallin P having less effect than the others. Table IV shows that the antioxidants also inhibited bleaching by pea seeds in the presence of oleic and palmitic acids.

Bleaching activity of seed extracts

(a) *The effect of various factors on the bleaching system.*—Table V shows the bleaching activity of seed extracts of three species with and without fatty acids added. White gram and hyacinth bean extracts, like soya extracts, had marked bleaching activity without added fatty acid, and it was sometimes so great that the addition of fatty acid caused little increase. Extracts from powders or soaked seeds of several different varieties of pea bleached little unless a fatty acid was added. Linoleic acid (or linolenic acid) always increased bleaching most with oleic acid next. The optimum pH for bleaching activity of extracts was near 6, the same as for ground cotyledons.

Without a seed extract present, fatty acids did not bleach chlorophyll suspensions with 4 min. shaking, but after long incubation some chlorophyll was degraded even though the pH of the system did not change.

Soya extracts kept at 0° retained their bleaching activity much longer than those made from peas. Most of the bleaching activity was destroyed by heating for 5 min. at 72°.

(b) *Course of the bleaching reaction.*—With an active enzyme preparation, the green colour of a chlorophyll-containing suspension rapidly disappeared and the absorption throughout the visible region greatly decreased (Fig. 5). Sometimes there was slight browning. When the

Table III

Effect of antioxidants on chlorophyll bleaching by soya-bean, pea and white gram seeds in the presence of linoleic acid
(for experimental conditions see text)

Components of reaction mixture	Bleaching, %			
	Pea 'Kelvedon Wonder'	Soya		White Gram
	2 seeds 2 min. grinding	2 seeds 2 min. grinding	3 seeds 4 min. grinding	1 seed 2 min. grinding
No linoleic acid, no antioxidant	3	26	52	3
Linoleic acid (10 mg.)	65	60	94	52
Santoquin (about 5 mg.)	8	13	<5	2
Linoleic acid and Santoquin	12	15	12	5
Progallin P (about 25 mg.)	2	8	7	2
Linoleic acid and Progallin P	18	29	53	24
NDGA[a] (10 mg.)	2	2	<2	2
Linoleic acid and NDGA[a]	2	4	<2	5
Linoleic acid and BHT[b] (10 mg.)	—	13	—	—

[a] = nordihydroguaiaretic acid [b] = butylated hydroxytoluene

Table IV

Effect of antioxidants on chlorophyll bleaching by pea seeds in the presence of oleic and palmitic acids
(conditions of experiment see text)

Amount of antioxidant in reaction mixture	Bleaching, %		
	No fatty acid added	Oleic acid	Palmitic acid
No antioxidant	3	71	46
Santoquin (5 mg.)	–	0	0
Progallin P (25 mg.)	–	3	11
NDGA (10 mg.)	–	0	3
BHT (10 mg.)	–	0	0

Table V

Chlorophyll bleaching activity of seed extracts in presence of various fatty acids

(The extract from pea var. 'Onward' was from dry powder, the other extracts were from soaked seeds. The reaction mixture contained 10 mg. of fatty acid)

Acid added	% Bleaching in 2 min.			
	Pea 'Peter Pan'	Pea 'Onward'	White gram	Hyacinth bean
No fatty acid	12	9	21	27
Linoleic acid	38	48	53	46
Oleic acid	26	42	41	35
Ricinoleic acid	19	33	29	29
Stearic acid	26	26	35	30
Elaidic acid	—	38	—	—
Elaeostearic acid	—	28	—	—

reaction was continued for several minutes a sample diluted with acetone was pale yellow and gave no peak in the red region of the spectrum.

The shape of the absorption curve of partly bleached chlorophyll suggested that, in a mixture of chlorophylls *a* and *b*, the *b* component was bleached more slowly than the *a* and this was confirmed by paper chromatography. A preparation of partly bleached chlorophyll in aqueous acetone was transferred to ether and chromatographed with a solvent mixture made up with light petroleum 4; benzene 1: acetone 0·5 (Holden[17]). Comparison with the unbleached preparation showed that about 80% of the chlorophyll *a* had disappeared but only about 40% of the chlorophyll *b*.

Coloured degradation products of the chlorophylls such as pheophytins, pheophorbides and chlorophyllides were not detected on chromatograms. All these compounds were readily bleached under similar conditions to those required for chlorophyll *a* indicating that neither magnesium nor phytol is important in the bleaching reaction.

Carotene was bleached by all the legume seed extracts tested, not only in the presence of linoleic acid but also of oleic acid.

(c) *Preliminary steps in separating the bleaching factor.*—Fractionation of crude soya and pea extracts with ammonium suphate showed that the material responsible for bleaching chlorophyll was precipitated between 30% and 55% saturation. Much of the activity was retained on prolonged dialysis against distilled water. Fractionation with acetone led to complete inactivation.

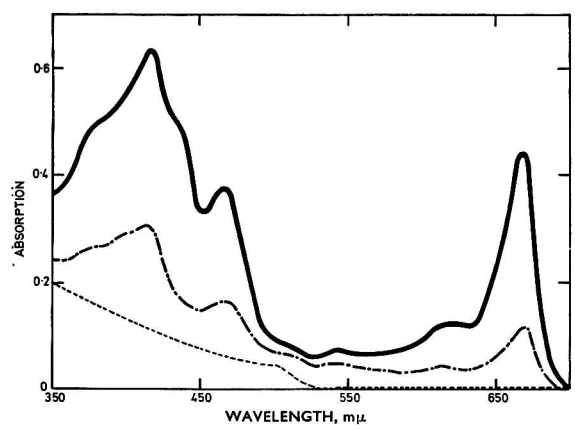

FIG. 5.—*Absorption curves of a mixture of chlorophylls* a *and* b *in acetone before and after shaking with a pea-seed extract and linoleic acid*

———— before shaking
——·—— 4 min. shaking with 0·2 ml. of pea-seed extract
– – – – – 4 min. shaking with 0·5 ml. of pea-seed extract

Formation of hydroperoxides by seed extracts

When linoleic acid was incubated with a seed extract, hydroperoxide was rapidly formed over a wide pH range. Oleic hydroperoxide was also formed under similar conditions but the amount was much smaller. Fig. 6 shows that, to obtain the same hydroperoxide value at 1 min., eight times more pea-seed extract was needed with oleic than with linoleic acid.

The optimum pH for linoleate hydroperoxide formation by pea and soya extracts (pH 7·5 — 8·0) was a little lower than for a commercial preparation of purified lipoxidase under the same conditions (pH 8·5). Crude extracts from soya flour (5 ml. of acetate buffer solution/g. of flour) formed hydroperoxide at a rate equivalent to that of a solution of about 20 g./l. of commercial lipoxidase.

Fig. 7 compares the formation of linoleate hydroperoxide and chlorophyll bleaching by a soya extract at pH 5·9 and pH 8·0. Although hydroperoxide is formed faster at the higher pH, the bleaching is much less. At pH 8 the amount of hydroperoxide formed is almost linearly proportional to the time of reaction, but at pH 5·9 the formation slows with time. A much greater volume of seed extract was required to obtain chlorophyll bleaching than was necessary to form linoleate hydroperoxide rapidly.

Preliminary experiments indicated that with amounts of seed extract of the same order as those used for chlorophyll-bleaching experiments, there was a complicated relationship between the amount of linoleate hydroperoxide formed and the time of reaction. With small volumes of extract and a short reaction time, the peroxide formed was proportional to the volume of extract. But with larger volumes, the amount of hydroperoxide in the reaction mixture was actually less than with a small volume, as shown in Fig. 8 with an extract from the powder of Feltham First peas. With increasing reaction time, the amount of hydroperoxide rose rapidly to a maximum and then slowly declined. This fall in peroxide content was seen clearly with soya extracts but it was neither so large nor so rapid as that found by Gini & Koch;[15] with pea extracts it was even smaller. When the reaction mixture was boiled after some hydroperoxide had been formed, there was an immediate drop in the hydroperoxide value but no further change on prolonged incubation.

Table VI compares the lipoxidase activity with the chlorophyll-bleaching activity, in the presence of linoleic acid, of extracts from seeds of some of the same species as in Table II. The formation of hydroperoxide was measured with several different amounts of each extract in the range where the amount of hydroperoxide formed was approximately proportional to the volume of seed extract used. Several species had considerably less lipoxidase activity but similar bleaching activity to that of soya.

Fig. 9 compares the ultra-violet absorption spectrum of linoleic acid, shaken for 2 min. at pH 6 with a pea-seed extract, with that for linoleic acid plus purified lipoxidase at pH 8. In the latter case there was one peak in the ultra-violet region with a maximum near 235 mμ, because of the formation of conjugated diene hydroperoxide. With the pea extract, there was a peak in the same place and also a much smaller one with a maximum

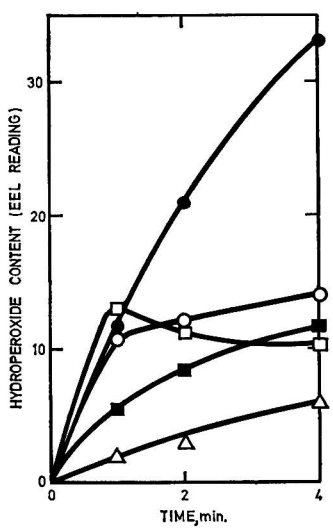

FIG. 6.—*Comparison of hydroperoxide formation with oleic and linoleic acids by the action of a pea-seed extract*

● linoleic acid, 0·05 ml. pea extract
△ oleic acid, 0·05 ml. pea extract
■ oleic acid, 0·1 ml. pea extract
○ oleic acid, 0·4 ml. pea extract
□ oleic acid, 0·6 ml. pea extract

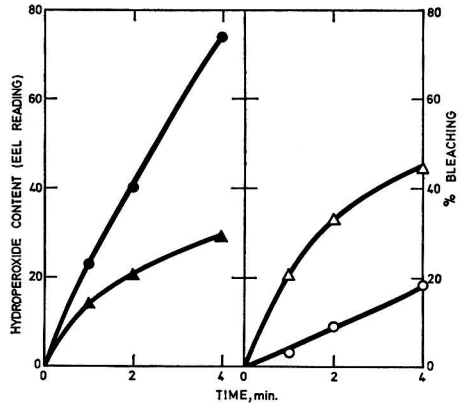

FIG. 7.—*Comparison of linoleate hydroperoxide formation and chlorophyll bleaching by a soya flour extract at pH 5·9 and pH 8·0*

Hydroperoxide formation: ●, 0·025 ml. extract, pH 8·0; ▲, 0·025 ml. extract, pH 5·9. Chlorophyll bleaching: ○, 0·4 ml. extract, pH 8·0; △, 0·4 ml. extract, pH 5·9

near 280 mμ. Oleic acid shaken with a pea extract for 4 min. showed a small increase in absorption between 215 and 250 mμ but no peaks. This is not unexpected as oleate hydroperoxides do not absorb between 220 and 350 mμ.

Effect of peroxidation of linoleic acid

Chlorophyll was not bleached under the conditions of the test by peroxidised linoleic acid without seed extract. With extract present there was no increase in the bleaching, and in some experiments a small decrease, when peroxidised linoleic acid was added instead of a fresh solution. The linoleic acid was usually peroxidised by incubating with commercial lipoxidase at 20° overnight. Under these conditions the lipoxidase was inactivated and did not affect the subsequent bleaching when seed extract and chlorophyll were added. When, however, the

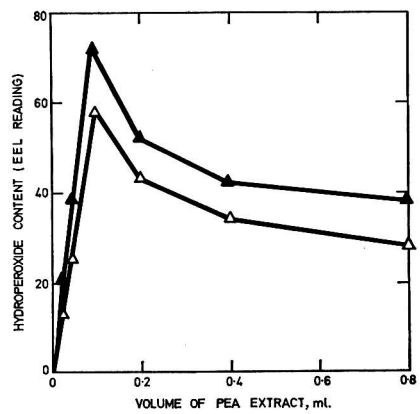

FIG. 8.—*Relationship between linoleate hydroperoxide formation and volume of pea-seed extract during a short incubation period*

△ 2 min. incubation ▲ 4 min. incubation

Table VI

Comparison of chlorophyll-bleaching activity with linoleic acid present and lipoxidase activity in extracts from seeds

	Lipoxidase activity	Chlorophyll-bleaching activity		Lipoxidase activity	Chlorophyll-bleaching activity
Soya	100	100	Pea	22	86
Black gram	41	65	Green gram	19	44
Hyacinth bean (old seed)	38	88	Lupin	19	18
Black-eye bean	32	115	Broad bean	17	40
White gram	29	104	Groundnut	4	3
French bean	26	54			

linoleic acid was peroxidised for only a short period it was essential to inactivate any residual lipoxidase by boiling, because this enzyme, in the presence of seed extracts, greatly increased chlorophyll bleaching. This is discussed more fully in a later section.

Effect of octan-2-ol

Mapson & Moustafa[2] found that the lipoxidase activity of extracts from ungerminated pea seeds could be initiated by adding alcohols such as octan-2-ol, which altered the condition of the fatty acid substrate that was also present in the extract, making it accessible to the enzyme.

Adding octanol sometimes increased the amount of chlorophyll bleached by ungerminated soaked seeds without also adding a fatty acid to the system, but the results were erratic. However, octanol increased the rate of bleaching by extracts from pea seeds without extra fatty acid, but inhibited the reaction in presence of added fatty acid. With soya flour extracts octan-2-ol inhibited bleaching with or without fatty acid added. The formation of linoleate hydroperoxide by extracts from ungerminated peas was strongly inhibited by octan-2-ol.

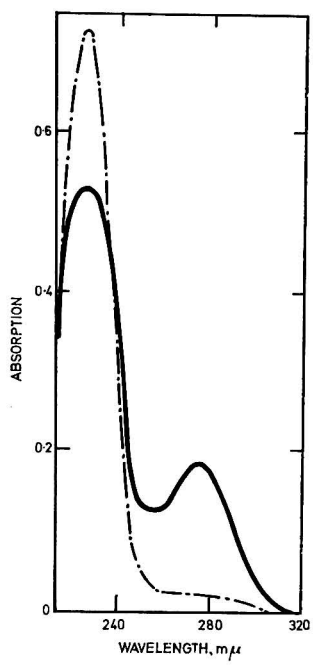

FIG. 9.—*Ultraviolet absorption curves of linoleic acid after the action of either lipoxidase or a pea-seed extract for 2 min.*

—·— lipoxidase, pH 8 ——— pea-seed extract, pH 6.

Effect of cyanide

Mapson & Moustafa[2] distinguished two factors in pea extracts involved in the oxidation of glutathione, based on their sensitivity to cyanide. Lipoxidase is comparatively insensitive to cyanide and 0·002 M does not inhibit. To test the bleaching reaction for cyanide-sensitivity an increased amount of buffer solution in the reaction mixture was required to maintain the pH at 6. At this pH and in a mixture which is being shaken the concentration of cyanide is only approximate, but with potassium cyanide at about 0·002 M bleaching was not inhibited.

Effect of Tween 20

Surrey[22] used the detergent Tween 20 to dissolve linoleic acid for lipoxidase determinations. He found that, with 0·25% linoleate and 0·25% Tween 20 in the

reaction mixture, purified lipoxidase was considerably more active over a wide pH range than without detergent. In the present investigation, with a linoleic concentration of 0·02%, hydroperoxide formation by a pea extract was increasingly stimulated by concentrations of Tween up to 0·04%, above this the stimulation diminished and above 0·06% the detergent was inhibitory. Tween did not affect chlorophyll-bleaching activity in the same way. All concentrations tested caused some inhibition; at 0·05% bleaching was only about 30% of that without detergent present and at 0·1% there was no bleaching.

Experiments with purified lipoxidase

Preparations of commercial purified lipoxidase tested under the same conditions as seed extracts caused little or no bleaching of chlorophyll. For example, 10 mg. of a preparation from Seravac Laboratories (batch E4) in the standard reaction mixture with linoleic acid, gave less than 20% bleaching in 4 min. at pH 6, but neither raising the pH to 8·5 nor lowering the acetone concentration increased the bleaching. In contrast, carotene appeared to be bleached by the purified lipoxidase under a fairly wide range of conditions, but this was not studied in detail.

Although the purified lipoxidase apparently did not bleach chlorophyll by itself, it greatly increased bleaching when added with linoleic acid to a seed extract. Table VII shows that addition of lipoxidase to a pea-seed extract without linoleic acid gave some increase in bleaching of chlorophyll, but addition of the acid gave a much enhanced activity. A similar, but smaller, effect was obtained with oleic acid. Lipoxidase added to a boiled pea extract or a diffusate from a fresh extract gave no bleaching.

Table VIII shows that when a soya extract was incubated for a short period with linoleic acid before the chlorophyll was added, the bleaching was considerably diminished and after a

Table VII

Effect of adding purified lipoxidase on the chlorophyll-bleaching activity of a pea extract

Components of reaction mixture	Bleaching, %		
	1 min.	2 min.	4 min.
Pea extract 0·2 ml., no fatty acid	0	5	14
Pea extract 0·2 ml., linoleic acid 10 mg.	16	27	38
Boiled pea extract 0·2 ml., linoleic acid 10 mg.	0	0	0
Lipoxidase 1 mg., linoleic acid 10 mg.	0	0	0
Lipoxidase 2 mg., linoleic acid 10 mg.	0	3	5
Pea extract 0·2 ml., no fatty acid, lipoxidase 1 mg.	3	14	23
Pea extract 0·2 ml., linoleic acid 10 mg., lipoxidase 1 mg.	40	57	70
Pea extract 0·2 ml., linoleic acid 10 mg., lipoxidase 2 mg.	52	74	—
Pea extract 0·2 ml., oleic acid 10 mg.	14	22	31
Pea extract 0·2 ml., oleic acid 10 mg., lipoxidase 2 mg.	20	30	46

Table VIII

Effect on the bleaching factor of incubating a soya-flour extract with linoleic acid

	Bleaching, %		
	1 min.	2 min.	4 min.
Soya extract (0·4 ml.) with linoleic acid and chlorophyll, no pre-incubation	22	32	43
Soya extract (0·4 ml.) with linoleic acid, chlorophyll and 1 mg. lipoxidase, no pre-incubation	33	53	73
Lipoxidase (1 mg.), linoleic acid and chlorophyll	0	1	3
Soya extract (0·4 ml.), 3 min. pre-incubation with linoleic acid before chlorophyll added	16	22	34
Soya extract (0·4 ml.), 3 min. pre-incubation before chlorophyll and lipoxidase (1 mg.) added	22	34	53
Soya extract (0·4 ml.), 9 min. pre-incubation before chlorophyll added	12	19	29
Soya extract (0·4 ml.), 9 min. pre-incubation before chlorophyll and lipoxidase added	17	28	42
Soya extract (0·4 ml.), 27 min. pre-incubation before chlorophyll added	0	0	0
Soya extract (0·4 ml.), 27 min. pre-incubation before chlorophyll and lipoxidase added	0	0	0

longer period of incubation there was no bleaching at all because the bleaching factor was inactivated. Adding lipoxidase still increased bleaching after 9-min. pre-incubation of linoleic acid and soya extract, but after 27 min. did not do so. But pre-incubation also has another effect; linoleic acid after treatment with seed extract was only about 2/3 as effective in the bleaching system as the fresh acid or acid peroxidised by lipoxidase.

Addition of lipoxidase to linoleic acid and ground cotyledons of groundnut and broad bean which had low lipoxidase- and bleaching-activities did not significantly increase the bleaching.

Investigation of possible bleaching action of haematin compounds

Hawthorn & Todd[23] found that minute amounts of catalase added to a system containing carotene and slightly peroxidised linoleic acid caused coupled bleaching of carotene, but there was no such reaction with peroxide-free linoleic acid. Catalase added to the chlorophyll-bleaching system containing pea extract had no effect at a concentration of 2 mg./l. in the reaction mixture and at 40 mg./l. increased the bleaching by less than 20%. Peroxidised linoleic acid instead of fresh acid also had no effect. Addition of catalase to purified lipoxidase, each enzyme at 40 mg./l. in the reaction mixture, did not increase the trace of bleaching given by lipoxidase alone.

Horseradish peroxidase at 2 mg./l. in the reaction mixture did not increase the bleaching by pea extract with either fresh or peroxidised linoleic acid. Horseradish root ground with chlorophyll gave no bleaching either with or without lipoxidase.

Discussion

Lipoxidase activity has been implicated in the degradation of chlorophyll in frozen green vegetables,[6,7] but the fact that various long-chain fatty acids, in addition to those that are substrates for lipoxidase, caused chlorophyll to be bleached when added to ground legume seeds suggested that other factors were also involved. In extracts from seeds, the chlorophyll-bleaching activity, although clearly bearing some relation to lipoxidase activity, did not parallel it exactly. Also, the pH optimum for bleaching was much lower than for peroxidation of linoleic acid by seed extracts. Purified lipoxidase did not bleach chlorophyll under the conditions used for testing seed extracts. This does not agree with the results obtained recently by Walker,[7] who stated, without giving experimental details, that the effect on chlorophyll of lipoxidase (from Sigma Chemical Co.) was the same as that of an extract from French beans. He found that, when a bean extract was incubated with a crude chlorophyll preparation and linoleic acid, the chlorophyll was first broken down to pheophytin under both aerobic and anaerobic conditions. After a lag period of some minutes the pigments were bleached aerobically but not anaerobically. With purified chlorophyll there was little or no conversion to pheophytin and there was no lag period before chlorophyll colour started to disappear. Under the conditions used in the present work, no difference between crude and purified chlorophyll was noticed, both being bleached extremely rapidly, and pheophytin and pheophorbide were not detected. This does not rule out the possibility of coloured breakdown products sometimes being formed, particularly when conditions are not optimal.

No attempt has been made to identify the colourless breakdown products formed in the chlorophyll-bleaching reaction. It may be that the porphin ring is still intact and the products are colourless, as are porphyrinogens,[24] because of interruption of the conjugated double-bond system present in chlorophyll. However, the porphin ring may have been opened giving rise to compounds such as ethyl-methylmaleimide and haematinic acid imide, which are formed on oxidation with chromic acid. This is to be investigated. There is no evidence that in the chlorophyll-bleaching system chlorophyllase action precedes loss of colour.

Work by Gini & Koch[15] on a lipoperoxidase in soya extracts and by Blain & Styles[12] and Blain & Barr[13] on the bleaching of carotene during hydroperoxide breakdown catalysed by this enzyme, suggested that chlorophyll might be bleached by a similar factor. The breakdown of lipid peroxides is catalysed by a range of agents, notably by haem compounds in very low concentration.[25] A mechanism for the haematin-catalysed decomposition of peroxide involving the formation of free-radicals has been given by Tappel,[26,27] who considers that haematin

catalysis is non-specific and the only requirement is that unsaturated compounds should form peroxide during the course of the reaction. Freeman[28] found that the decomposition of labelled linoleic acid hydroperoxide by cytochrome *c* was not affected by boiling, but that a hydroperoxide breakdown-system in homogenised intestinal mucosa was heat labile. O'Brien[29] found a similar system in liver mitochondria. These systems seem to be similar to the one in legume-seed extracts.

In addition to the breakdown of linoleate hydroperoxide measured by the decrease in absorption in the ferric thiocyanate method, the formation of substances absorbing at 280 mμ when linoleic acid was incubated with a seed extract indicates breakdown products of hydroperoxides such as carbonyl compounds.[26] Siddiqi & Tappel,[30] when studying the action of pea lipoxidase on linoleic acid, found this peak in the absorption spectrum of the oxidation products and suggested that the hydroperoxides were unstable and gave rise to many other oxygen-consuming reactions.

Chlorophyll bleaching required much more seed extract than was needed to form hydroperoxide rapidly. Yet addition of a small amount of lipoxidase, forming hydroperoxide at only 10% of the rate of the seed extract to which it was added, greatly increased bleaching in the presence of linoleic acid. This effect might depend on a thermolabile factor in the seed extract that is necessary for co-oxidation of chlorophyll during lipoxidase action. It could be that some factor other than lipoxidase is present in the enzyme preparation. However, it is more likely that the lipoxidase is stimulating one stage of a series of reactions that are catalysed by factors in the seed extract. By increasing the formation of hydroperoxide more will be available to be broken down by the lipoperoxidase in the short period before it is inactivated.

Oleic acid is peroxidised by seed extracts and the peroxide appears to be broken down rapidly. Although much less peroxide is formed than with linoleic acid, it seems to be sufficient to start the chain of reactions during which the chlorophyll is bleached. The small increase of bleaching by lipoxidase in the presence of oleic acid is probably due to lipoxidase action on substrates present in seed extracts, because the enzyme increases bleaching by seed extracts when no fatty acid is added.

Other fatty acids, including saturated acids, form hydroperoxides of undetermined structure[31] but the reaction is usually extremely slow. Factors in legume seeds may catalyse the formation of enough peroxide from acids such as stearic to initiate the chain.

Soya-bean cotyledons and extracts made from them will bleach chlorophyll without extra fatty acid. This is presumably because they are particularly rich in lipoxidase and contain a suitable fatty acid substrate that is available for peroxide formation. In peas, fatty acid is not available in the cotyledons of ungerminated seeds but becomes so on germination.

Commercial antioxidants prevented chlorophyll bleaching by inhibiting peroxidation. Some concentrations of the detergent Tween 20 increased linoleate hydroperoxide formation but partly inhibited chlorophyll bleaching. Both effects are probably due to inhibition of peroxide decomposition. Mapson & Moustafa[2] found that octan-2-ol initiated lipoxidase activity in pea-seed extracts, but in the present work linoleate hydroperoxide formation was inhibited by octan-2-ol. There is no obvious explanation for this difference.

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Rothamsted Experimental Station,
Harpenden
Herts.

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THE *IN VITRO* AND *IN VIVO* OXIDATION AND HYDROLYSIS OF MALATHION BY WHEAT GRAIN ESTERASES

By D. G. ROWLANDS

Wheat grain esterases were shown to oxidise malathion to malaoxon which had no lasting inhibitory effect on those hydrolytic enzymes involved in the degradation of malathion. Breakdown of malaoxon proceeded by routes similar to those for malathion and it was concluded, from the small amount of malaoxon produced in a short storage trial, that oxidation played only a minor role in the degradation of malathion by wheat grain esterases.

Introduction

Previous work[1] on the breakdown of malathion by grain esterases (which included a diagram of probable degradation reactions) left unanswered the question whether an oxidative mechanism (i.e., P = S → P = O) was involved in this degradation or only hydrolytic pathways were followed. The present study was undertaken to determine whether malaoxon could be produced from malathion by the oxidases in grain and, if so, whether it affected the hydrolytic degradative systems previously shown to be present.

The grain used for the experiment was Manitoba wheat and the sample showed 85% germination at both the start and finish of the investigation.

Experimental

Oxidation of malathion to malaoxon in vitro

Four 20-g. lots of crushed wheat grains were extracted at room temperature by shaking with 100 ml. of acetone and freed from solvent in a vacuum desiccator, after filtration through a Buchner funnel. Three of the samples were shaken with 100 ml. of phosphate buffer solution, pH 7, for 1 h. at room temperature, then partitioned severally with 50 ml. of n-hexane, diethyl

ether or chloroform; the remaining sample was extracted with 50 ml. of 1 : 1 methanol and water, also at room temperature. After centrifugation at 1100 *g* to break emulsions, each organic extract was filtered, dried over sodium sulphate and evaporated. The residues were dissolved in 0·1 ml. of chloroform, and 20 μl. of the viscous solutions so obtained were spotted on Whatman No. 4 papers and developed for 8 min. with ethyl acetate. Further chromatography, with ethanol as ascendant phase at right angles, resolved the duplicated extractives seen under ultra-violet light into different components. These were marked in pencil, cut from the papers and digested in 1 ml. of chloroform. The 1-ml. chloroform extracts were stored at 0° until required, then added to tubes containing 0·5 μg. of malathion and incubated for 30 min. at 37°, with shaking. After incubation the chloroform was evaporated and each residue assayed for anti-cholinesterase activity as described previously.[1]

Carboxyesterase activity in wheat

One g. of crushed wheat was shaken for 30 min. at room temperature with 50 ml. of buffer solution (pH 7); a 25-ml. portion was decanted into a tube and an initial determination made of the pH. One ml. of a 1 : 1 mixture of ethyl maleate and acetone was added and the tube incubated for 1 h. at 37°. The pH was again determined and the decrease indicated the amount of free acid liberated by the esterase. No further hydrolysis occurred if the reaction time exceeded 1 h.

To ascertain the effect of malaoxon on the carboxyesterase, a 0·5-μg. residue of malaoxon was dissolved in 25 ml. of the buffered wheat extract and the vessel incubated at 37° for 30 min. before determining the pH. The reaction was continued as described above.

Acid-phosphatase degradation of malathion

One g. of crushed wheat was shaken with 50 ml. of phosphate buffer solution (pH 5·5) for 30 min. at room temperature. From the resulting extract 25 ml. were decanted into a tube containing 100 μg. of malathion. The tube was shaken during incubation at 37° for 30 min. then immersed in a freezing mixture of ice and salt to halt any further reaction. While still cold the solution was twice partitioned with 25 ml. of chloroform after acidification of the aqueous phase to pH 2 with hydrochloric acid; the chloroform extracts were evaporated to a thin film which was dissolved in 20 ml. of hexane. The hexane solution was run through a 20-g. column of acid-washed alumina (Brockman activity III) under suction and the column was drained before elution with (*a*) 20 ml. of diethyl ether and (*b*) 20 ml. of acetonitrile. (Dimethyl phosphorothiolothionate, one of the products, is unstable on this adsorbent if left in contact longer than 30 min.)

The hexane portion, containing those metabolites not held on the column—and dimethyl phosphorothionate in particular—was concentrated to 1 ml. Two drops of a 1 : 1 mixture of ethanol and 1N-hydrochloric acid were added, followed by 1 ml. of a 0·1% w/v solution of 2,6-dibromo-*N*-chloroquinonimine (DCQ) in cyclohexane.[2] The vessel was swirled, lightly corked and placed on an oil bath, maintained at 100°, for exactly 12 min. The residue was cooled, dissolved in ethanol and the solution made up to 10 ml. before determination of the optical density at 425 mμ. Calibration graphs were prepared from pure dimethyl phosphorothionate in the 0–30 and 30–150 μg. ranges. Specimen calibration graphs are shown in Fig. 1.

The diethyl ether fraction was treated in analogous fashion except that the optical density was determined at 520 mμ and the reference compound was pure dimethyl phosphorothiolothionate, freshly liberated from its sodium salt. Intact malathion in the acetonitrile eluate was determined similarly, absorption being measured at 520 mμ; the figures obtained were checked for accuracy by a modification of the method of Norris *et al.*[3] DCQ reacts only with P = S compounds.

To determine the effect of malaoxon on the acid-phosphatase action, residues of 0·5, 5·0 and 50 μg. of malaoxon were dissolved in 25 ml. of buffered wheat extract and incubated at 37° for 30 min. Each solution was then added to a tube containing 100 μg. of malathion, incubated for 30 min. at 37° and chilled in freezing mixture. Partitioning, chromatography and determination of phosphatase products and intact malathion were as described above.

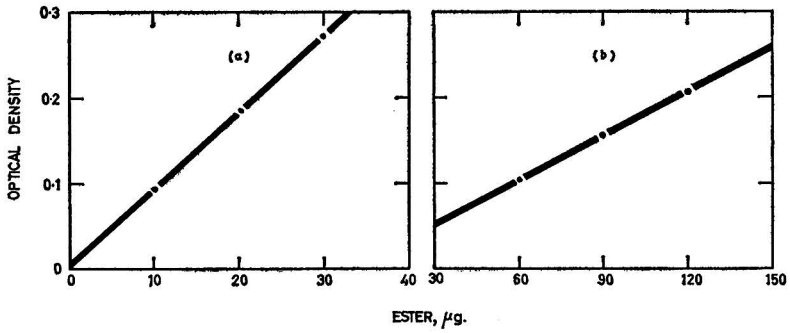

FIG. 1.—*Reaction of dimethyl phosphorothiolothionate with DCQ reagent*

(*a*) 0–30 μg. range. 0·1% w/v DCQ. 2 cm. cell at 520 mμ.
(*b*) 30–150 μg. range. 0·5% w/v DCQ. 1 cm. cell at 520 mμ.

Oxidation and hydrolysis of malathion in vivo

Twenty-five-g. samples of wheat, crushed to facilitate rapid penetration of the insecticide, were weighed into screw-capped jars and dosed with 250 μg. of malathion dissolved in 5 ml. of hexane. The solvent was evaporated and one sample was checked for anticholinesterase activity to ascertain that no malaoxon had been produced during the evaporation. The jars were then sealed and stored in the dark at 20°. Samples were withdrawn at 1, 6, 12, 48 and 168 h. and stored at −10° to prevent any further enzymic action. Control samples were dosed with 250 μg. of malathion immediately prior to extraction.

The production of malaoxon was assessed by measuring the increase in anticholinesterase activity shown over the experimental period. Those malaoxon metabolites with inhibitory properties were identified by the paper-chromatographic technique of Getz & Friedman.[4] For both these determinations samples were shaken with water for 1 h., the extract was acidified to pH 1 and twice partitioned with 50 ml. of chloroform; such extracts were dried with sodium sulphate and evaporated. Cholinesterase activity was measured as described previously.[1]

For identifying metabolites of malaoxon, several chloroform extracts were combined, evaporated, dissolved in 50 ml. of hexane and passed through a column of acid-washed alumina (activity V), draining the column and washing through with more hexane. The solvent was evaporated, the residues dissolved in 0·1 ml. of chloroform and applied to papers as described by Getz & Friedman.[4] R_F values so obtained were compared with those for pure malaoxon and synthesised metabolites.[1]

Results

Enzymic oxidation of malathion to malaoxon in vitro

Three of the seven extractives isolated were able to effect some conversion of P = S to P = O as shown by significant increases in anti-cholinesterase activity, but only one gave reproducible results with almost complete conversion of 0·5 μg. of malathion to malaoxon, with negligible hydrolytic degradation. This was a fraction, R_F 0·65 under the conditions used, obtained from chloroform, ether and aqueous methanol extracts. None of the 'oxidative' extractives showed any anti-cholinesterase activity themselves.

Effect of malaoxon on the carboxyesterase

Apparently malaoxon had no inhibitory effect on the *in vitro* liberation of free acid from ethyl maleate by a wheat-grain carboxyesterase, even when present at a level of 100 p.p.m. Pre-treatment of the grains with malathion or malaoxon, at levels ranging from 10–100 p.p.m., 1 day and 6 days before extraction did not alter the results (see Table I).

Table I

The in vitro *effect of malaoxon upon acid-phosphatase degradation of malathion in wheat grains*

Malaoxon applied, μg.	Malathion applied, μg.	P = S degradation products from phosphatase action			
		Intact malathion, μg.	Dmtpa*, μg.	Dmdtpa†, μg.	Balance**, μg.
Nil	100	20	43	22	15
0·5	100	20	43	25	12
5·0	100	20	46	22	12
50	100	20	42	24	14

* dimethyl phosphorothionate
† dimethyl phosphorothiolothionate
** probably dimethyl phosphate or simple phosphoric acids. Not detected.

The in vivo *oxidation and hydrolysis of malathion*

Table II shows that only small amounts of active inhibitors were found over a period of 1 week, rising to the greatest level between 1 and 2 days after treatment. Metabolites were identified when present in sufficient quantity to cause significant inhibition of cholinesterase, but identification was somewhat tentative due to the poor resolution of the less-potent inhibitors.

Table II

Anticholinesterase metabolites produced in vivo *by wheat grain esterases from malathion applied at* 10 *p.p.m.*

Time after treatment, h.	Inhibition of cholinesterase, %	Metabolites identified	*R_F of metabolites
1	Nil	Nil	Nil
6	2·9	malaoxon	1·0
12	14·0	malaoxon, dmtpa	1·0, 0·4
24	22·9	malaoxon, dmtpa	1·0, 0·4
48	12·8	dmtpa, monoacid, diacid, dmpa	0·4, 0·2 0·1 (0·08),
168	Nil	Nil	Nil

* approximate values for R_F due to poor resolution of the metabolites with weaker anticholinesterase activity
dmtpa = dimethyl phosphorothiolate
dmpa = dimethyl phosphoric acid
monoacid = *OO'*-dimethyl *S*-(1-carboxy-2-ethoxycarbonyl)ethyl phosphorothiolate
diacid = *OO'*-dimethyl *S*-(1,2-dicarboxy)ethyl phosphorothiolate

Discussion

The results showed that malathion could be converted to malaoxon by living wheat grains. Malaoxon so produced, being an active anti-esterase, might conceivably phosphorylate hydrolytic enzymes present, thus becoming undetectable, i.e., oxidation might initially increase breakdown of malathion by enhancing the electrophilic nature of the phosphorus atom but would eventually retard or prevent this breakdown. Greater lability of malaoxon, compared with malathion, would probably be more than counterbalanced by the inhibition of the degrading enzymes.

An organophosphate acts as an electrophilic agent in phosphorylating an enzyme; any change, therefore, that would increase its electrophilic properties, e.g., conversion of P = S to P = O, would improve the anti-esterase properties of the molecule. Such enhancement of the electrophilic nature of the ester also increases susceptibility to hydrolysis. Kojima & Ishizuka[5] have suggested a mechanism for the phosphorylation of carboxyesterases by malaoxon, but malaoxon is itself a carboxy-ester and, moreover, one that is very readily hydrolysed, so that degradation could occur before phosphorylation took place. Cook *et al.*[6] found that certain organophosphates partially or completely inhibited ' malathionase ' (malathion-carboxyesterase) *in vitro*, whilst Koivistoinen[7] showed similar effects with parathion and paraoxon in certain plants. None of the compounds they used contained a carboxyester grouping however.

Before the formation of an ' inhibited complex ' can occur at an enzyme site an organophosphate has to be hydrolysed by the enzyme, usually at the P–S- (or P–O-) bond, and if the substituents on the phosphorus atom are sufficiently nucleophilic the complex will be irreversible and stable to further (degradative) hydrolysis. If the substituents should be strongly electrophilic then hydrolysis can rapidly occur to lower phosphorus derivatives, thus liberating the enzyme once again.

When considering the interaction of carboxyesterase and malaoxon however, esteratic scission will be preferentially at the carboxylate link rather than the P–S- bond; the resulting metabolite(s) will then be less liable to further hydrolysis than their parent compound owing to the nucleophilic effect of the carboxyl anions. Although these metabolites are less effective inhibitors than malaoxon their effective phosphorylation might be more permanent because of their increased resistance to hydrolysis. However scission of the P–S-link would still have to occur before inhibition could take place and the nucleophilic anions would tend to prevent this happening.

Results obtained *in vitro* showed no inhibitory effect on a carboxyesterase-type hydrolysis even when the grains were pre-treated with malathion or malaoxon before extraction and addition of the substrate. That hydrolysis to malaoxon mono- and di-acids occurred *in vivo* is apparent from Table II. Since these carboxyesterase products were not detected in significant quantity until 48 h. after treatment, whereas malathion was degraded by the phosphatase and carboxyesterase action simultaneously and malaoxon phosphatase metabolites were detected after 12 h., it seems possible that a reversible phosphorylation may have taken place, with consequent reactivation of the enzyme. Hydrolysis of phosphorylated enzyme to enzyme plus phosphate is a chemical reaction dependent on time and temperature; a naturally-occurring reactivating mechanism has been observed (for cholinesterase) in mammalian serum[8] and, *in vivo*, in houseflies.[9]

Phosphorylation of the active phosphatase centres was also a possible reaction though there is no published evidence for inhibition of these enzymes by thiolates. Of the phosphomonoesterases, those likely to be present in wheat grains are acid phosphatases acting optimally in a pH range of 5–6 but, as Table I shows, malaoxon did not interfere with malathion degradation by these enzymes *in vitro* and metabolites were rapidly produced *in vivo* 6–12 h. after treatment of the grains.

When wheat grains were dosed with malathion at 10 p.p.m. and stored, anticholinesterase activity at the highest level recorded (Table II) was equivalent to that of only 0·3 μg. of malaoxon. As chromatography showed, other inhibitory compounds were undoubtedly contributing to this equivalent figure also and, being less electrophilic and therefore less potent inhibitors, must have been present in larger quantities; they could, however, have accumulated only from malaoxon produced initially. Even so it would seem that oxidation cannot play a very great part in initiating the enzymic breakdown of malathion in wheat grains.

Conclusion

Malathion applied to stored wheat grains is degraded enzymically to non-toxic P = S metabolites but a little oxidation does occur, followed by rapid degradation to the thiolo-analogues of the P = S compounds.

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Agricultural Research Council
Pest Infestation Laboratory
London Road
Slough, Bucks.

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SYNTHESIS AND INSECTICIDAL ACTIVITY OF *N*-METHYLENEFLUOROACETAMIDE DERIVATIVES

By M. PIANKA and D. J. POLTON*

Several *N*-methylenefluoroacetamides were synthesised and tested for contact (2 days) and systemic (7 days) activity against *Aphis fabae*. The contact activity seemed to depend on the ease of hydrolysis to fluoroacetamide. Generally, the systemic activity of the compounds was similar to that of fluoroacetamide, indicating that, during the longer period allowed for the systemic tests, hydrolysis to fluoroacetamide took place as the first step. Some compounds were too stable and showed little contact or systemic activity. The acaricidal activity and mammalian toxicity were also determined. A hydrolysis path is indicated, and the greater stability of certain derivatives is discussed.

Introduction

The insecticidal activities of sodium fluoroacetate[1,2] and of fluoroacetamide[3,4] are well known. We became interested during the 1950's in the synthesis and the biological activity of variously substituted *N*-methyl- and *N*-(2,2,2-trichloroethyl)-fluoroacetamides (**I**) of variously linked bis-*N*-methyl- and *N*-(2,2,2-trichloroethyl)-fluoroacetamides (**II-V**), and of other related fluorine compounds (**VI; VII**).

$FCH_2{\cdot}CO{\cdot}NH{\cdot}CHRR'$ (**I**)

$(FCH_2{\cdot}CO{\cdot}NH)_2X$ (**II**)

$(FCH_2{\cdot}CO{\cdot}NH{\cdot}CHR)_2X$ (**III**)

$(FCH_2{\cdot}CO{\cdot}NH{\cdot}CH_2{\cdot}O{\cdot}CH_2{\cdot}CH_2)_2$ (**IV**)

$[FCH_2{\cdot}CO{\cdot}NH{\cdot}CHR{\cdot}S{\cdot}CS{\cdot}NH{\cdot}CH_2]_2$ (**V**)

$FCH_2{\cdot}CO{\cdot}NH{\cdot}CH{:}CCl_2$ (**VI**)

$FCH_2{\cdot}CH_2{\cdot}O{\cdot}CH_2{\cdot}S{\cdot}CS{\cdot}NMe_2$ (**VII**)

Preparative methods

The compounds were synthesised by two methods. Method (I) depended on the acid-catalysed reaction of *N*-hydroxymethylfluoroacetamide with an alcohol to a fluoroacetamidomethoxyalkane, and with a mercaptan to a fluoroacetamidomethylthioalkane. Method (II) involved the reaction of *N*-chloromethyl- or *N*-(1,2,2,2-tetrachloroethyl)-fluoroacetamide with various nucleophiles.

* Present address: ' Shell ' Research Ltd., Woodstock Agricultural Research Centre, Sittingbourne, Kent.

N-*Hydroxymethylfluoroacetamide* (Compound No. 1)

Fluoroacetamide (30·8 g.) and potassium carbonate (0·2 g.) were dissolved in 32 c.c. of an aqueous solution of formaldehyde (37·5% w/v) by warming (steam bath). Chloroform (200 c.c.) was added, the mixture heated under reflux and the water removed from the reaction-mixture by means of a Dean & Stark trap. When most of the water had been removed, *N*-hydroxymethylfluoroacetamide crystallised out. After cooling the compound was filtered off (40·9 g.; 95·6%) as white plates (from ethyl acetate), m.p. 99–100° (Found: N, 12·8. $C_3H_6FNO_2$ requires N, 13·1%).

Method Ia

To *N*-hydroxymethylfluoroacetamide (21·4 g.) was added a mixture of the appropriate alcohol (100 c.c.) and conc. hydrochloric acid (2 c.c.). The mixture was shaken occasionally, and the hydroxymethylfluoroacetamide slowly went into solution. When all of the solid had dissolved (24 h.), the solution was neutralised with aqueous sodium hydroxide, and the excess alcohol and water was distilled off. Distillation of the residue under reduced pressure gave colourless oils. When a solid was obtained, it was recrystallised.

Methods Ib and Ic

These were similar to Method Ia, but neutralisation was carried out with anhydrous sodium carbonate (Ib) or sodium bicarbonate (Ic). The salt was then filtered off and the filtrate worked up as before.

Methods Id and Ie

These were as Methods Ib and Ic respectively but 1·5 equivalents of alcohol were used.

Table I lists *N*-fluoroacetamidomethoxyalkanes, the methods of preparation and their physical characteristics.

Table I

N-*Fluoroacetamidomethoxyalkanes*, $FCH_2{\cdot}CO{\cdot}NH{\cdot}CH_2{\cdot}OR$

No. of compound	R	Method	M.p. or b.p./mm.	n^{20} of oil or appearance of solid	Yield, %	Formula	*N* Found, %	*N* required, %
2	Methyl	Ib	103–104°/21	1·4315	64·0	$C_4H_8FNO_2$	11·8	11·6
3	Ethyl	Ib	105–106/27	1·4342	46·6	$C_5H_{10}FNO_2$	10·4	10·4
4	Allyl	Ia	67–70/0·07	1·4514	54·7	$C_6H_{10}FNO_2$	9·5	9·5
5	Prop-2-ynyl	Ie	77–79/0·25	1·4638	31·1	$C_6H_8FNO_2$	9·5	9·7
6	2-Hydroxyethyl	Ib	126–127/0·09	1·4594	46·8	$C_5H_{10}FNO_3$	9·5	9·3
7	2-Methoxyethyl	Ia	84/0·07	1·4421	61·8	$C_6H_{12}FNO_2$	8·5	8·5
8	2-Ethoxyethyl	Ia	88/0·1	1·4415	48·4	$C_7H_{14}FNO_2$	7·8	7·8
9	2-n-Butoxyethyl	Ic	87–89/0·04	1·4425	5·4	$C_9H_{18}FNO_2$	6·7	6·8
10	2-(2-Methoxyethoxy)ethyl	Ic	111/0·1	1·4479	52·5	$C_6H_{12}FNO_4$	6·4	6·7
11	2-(2-Ethoxyethoxy)ethyl	Ia	116/0·2	1·4498	39·0	$C_8H_{16}FNO_4$	6·3	6·3
12	2-(2-n-Butoxyethoxy)ethyl	Ic	139/0·25	1·4488	32·2	$C_{11}H_{22}FNO_4$	5·7	5·6
13	2-Chloroethyl	Ia	110/0·15*		58	$C_5H_9ClFNO_2$	8·1 Cl 20·8	8·2 20·9
14	Benzyl	Ib	115/0·1	1·5210	45·8	$C_{10}H_{12}FNO_2$	7·0	7·1
15	2-Acetoxyethyl	Ia	135/0·04	1·4514	42·9	$C_7H_{12}FNO_4$	7·6	7·3
16	2-Acetamidoethyl	Id	125–126 (from ethyl acetate)	white needles	12·5	$C_7H_{13}FN_2O_3$	14·3	14·6
17	1,1-Dimethylprop-2-ynyl	Ib	46–48 (from di-isopropyl ether)	white plates	12·5	$C_7H_{12}FNO_2$	8·2	8·1
18	Cyclohexyl	Ib	48–50 (from petroleum, b.p. 30–40°)	colourless needles	20·7	$C_8H_{14}FNO_2$	7·5	7·4
19	1-Methylcyclohexyl	Ib	82–84/0·9–1·1	crystallised rapidly	24·8	$C_{10}H_{18}FNO_2$	6·7	6·9

* This compound crystallised out. White needles m.p. 43–44° (from petroleum, b.p. 40–60°)

N-(*Ethylthiomethyl*)*fluoroacetamide* (Compound No. 20)

N-Hydroxymethylfluoroacetamide (40 g.), ethanethiol (50 c.c.), conc. hydrochloric acid (5 c.c.) and ether (500 c.c.) were mixed and set aside for 24 h., by which time most of the solid had dissolved. The ether layer was separated and dried (sodium sulphate). On evaporation of the ether the *compound* (**I**; R = H, R′ = SEt) was obtained (21 g.; 37·1%) as white needles (from di-isopropyl ether), m.p. 45–47° (Found: N,8·9. $C_5H_{10}FNOS$ requires N,9·3%).

N-(*Chloromethyl*)*fluoroacetamide* (crude)

N-Hydroxymethylfluoroacetamide was gently warmed with 1·4 equivalents of thionyl chloride. After the evolution of gases had subsided, petroleum (b.p. 60–80°) was added, the mixture kept for 15 min. at 50°, then the excess thionyl chloride and solvent was distilled off at 50° (bath temperature) under reduced pressure. The *compound* (**I**; R = H, R′ = Cl), a white solid, was unstable and was freshly prepared when required.

N-(*Benzoyloxymethyl*)*fluoroacetamide* (Compound No. 21)

A solution of *N*-chloromethylfluoroacetamide (from 10·7 g. of *N*-hydroxymethylfluoroacetamide) in acetone (50 c.c.) was added to a stirred suspension of powdered sodium benzoate (21·6 g.), in acetone (100 c.c.), during 10 min. Stirring was continued for a further 1·5 h. After filtration the acetone was evaporated from the filtrate. The *compound* (**I**; R = H, R′ = $O{\cdot}CO{\cdot}C_6H_5$) was obtained (12 g.; 56·8%) as white prisms (from di-isopropyl ether), m.p. 96–98° (Found: N,6·5. $C_{10}H_{10}FNO_3$ requires N,6·6%).

In a similar manner as for Compound 21 were prepared from *N*-chloromethylfluoroacetamide the compounds shown in Table II from the reagents indicated.

N-(1,2,2,2-*Tetrachloroethyl*)*fluoroacetamide*

Fluoroacetamide (15·4 g.), anhydrous chloral (29·5 g.) and chloroform (200 c.c.) were heated under reflux for 2·5 h. On cooling, crude *N*-(1-hydroxy-2,2,2-trichloroethyl)fluoroacetamide (**I**; R = CCl_3, R′ = OH) crystallised out. Thionyl chloride (16 c.c.) was added to the mixture and the whole was heated on the steam bath under reflux for 3 h. Ligroin was added and the ligroin and the excess thionyl chloride were removed *in vacuo*. The residue was recrystallised from ligroin (100 c.c.). The *compound* (**I**; R = CCl_3, R′ = Cl) was obtained (32 g.; 71%) as white prisms, m.p. 77–79° (Found: N,5·7. $C_4H_4Cl_4FNO$ requires N,5·8%).

N-(1-*Methoxy*-2,2,2-*trichloroethyl*)*fluoroacetamide* (Compound No. 27)

Sodium (2·3 g.) was dissolved in methanol (100 c.c.) and to this solution was added a solution of *N*-(1,2,2,2-tetrachloroethyl)fluoroacetamide (24·3 g.) in acetone (25 c.c.). After filtration, the methanol and acetone were removed from the filtrate *in vacuo*. To the residue acetone was added (50 c.c.). After filtration, the acetone was removed from the filtrate *in vacuo* leaving an oil which solidified when kept (18·8 g.; 78·8%). The *compound* (**I**; R = CCl_3, R′ = OMe) was obtained as white needles (from petroleum), m.p. 53–55° (Found: N,6·0. $C_5H_7Cl_3FNO_2$ requires N,5·9%).

N-(1-*p-Chlorophenylthio*-2,2,2-*trichloroethyl*)*fluoroacetamide* (Compound No. 28)

In a similar manner a solution of sodium *p*-chlorobenzenethiolate (5·3 g.) in acetone (50 c.c.) was added a solution of *N*-(1,2,2,2-tetrachloroethyl)fluoroacetamide (7·7 g.) in acetone (50 c.c.). After 3 h. the mixture was filtered and the acetone distilled off *in vacuo*. The residual oil slowly

Table II

N- (*Substituted-methyl*) *fluoroacetamides* $FCH_2{\cdot}CO{\cdot}NH{\cdot}CH_2R$ *prepared from the reagents named in acetone solution*

Compound No.	Reagent and temperatures of reaction	Derivative obtained	R=	m.p., °c	Formula	Nitrogen, % Found	Nitrogen, % Required
22	Na *p*-chlorobenzenethiolate in alcohol, −20°	*N*-(*p*-chlorophenylthiomethyl)fluoroacetamide[a]	$S{\cdot}C_6H_4Cl$-*p*	73–74	$C_9H_9ClFNOS$	5·9	6·0
23	Na benzothiazole-2-thiolate, room temperature	*N*-(benzothiazol-2-ylthiomethyl) fluoroacetamide[b]	$S{\cdot}C_7H_4NS$	123–125	$C_{10}H_9FN_2OS_2$	10·7	10·9
24	Na dimethyldithiocarbamate (aqueous), −60 to −80°	*N*-(dimethyldithiocarbamoylmethyl)fluoroacetamide[c]	$S{\cdot}CS{\cdot}NMe_2$	77–77·5	$C_6H_{11}FN_2OS_2$	13·0	13·3
25	K thiocyanate, room temperature	*N*-(thiocyanatomethyl)fluoroacetamide[d]	SCN	77–77·5	$C_4H_5FN_2OS$	18·7	18·9
26	K phthalimide, room temperature	*N*-(phthalimidomethyl)fluoroacetamide[e]	phthalimido	187–189	$C_{11}H_9FN_2O_3$	11·9	11·9

[a] recrystallised from di-isopropyl ether
[b] recrystallised from ethyl acetate
[c] recrystallised from isopropanol
[d] recrystallised from carbon tetrachloride
[e] recrystallised from acetone

crystallised. The *compound* (I; R = CCl_3, R′ = S·C_6H_4·Cl-*p*) was obtained (5·8 g.; 52·3%) as white needles (from di-isopropyl ether), m.p. 88–89° (Found: Cl, 4·1. $C_{10}H_8Cl_4FNOS$ requires Cl,4·1%).

N-(1-*Dimethyldithiocarbamoyl-2,2,2-trichloroethyl)fluoroacetamide* (Compound No. 29)

This was prepared by treatment of *N*-(1,2,2,2-tetrachloroethyl)fluoroacetamide with an equivalent proportion of aqueous sodium dimethyldithiocarbamate in 2 volumes of acetone with vigorous stirring at −6o to −8o°, and the product obtained by concentration of the reaction mixture and recrystallisation of the concentrate from isopropanol. The *compound* (**I**; R = CCl_3, R′ = S·CS·NMe_2) was obtained (77%) as white prisms, m.p. 128·5–129° (Found: N,8·6. $C_7H_{10}Cl_3FN_2OS$ requires N,8·6%).

NN′-*Methylenedifluoroacetamide* (Compound No. 30)

N-Hydroxymethylfluoroacetamide (21·4 g.) was dissolved in conc. sulphuric acid (50 c.c.) and the solution set aside for 16 h. It was then poured on to crushed ice, the mixture neutralised with aqueous sodium hydroxide and evaporated to dryness under reduced pressure. The dry residue was then extracted with acetone. On evaporating the extract the *compound* (**II**; X = CH_2) was obtained (5 g.; 17·2%) as white needles (from xylene), m.p. 164–165° (Found: N,16·8. $C_5H_8F_2N_2O_2$ requires N,16·9%).

Bis(fluoroacetamidomethyl) ether (Compound No. 31)

To a solution of *N*-chloromethylfluoroacetamide (from 10·7 g. of *N*-hydroxymethylfluoroacetamide) in acetone (100 c.c.) was added anhydrous sodium carbonate (10 g.). When the evolution of carbon dioxide subsided, the mixture was stirred for a further hour at 70° and filtered. The white solid that was obtained on concentrating the filtrate was recrystallised from ethyl acetate. The *compound* (**III**; R = H, X = O) was obtained as small white needles, m.p. 90–92° (Found: N,14·4. $C_6H_{10}F_2N_2O_3$ requires N,14·4%).

1,4-*Bis(fluoroacetamidomethoxy)butane* (Compound No. 32)

N-Hydroxymethylfluoroacetamide (21·4 g.) was added to a mixture of butane-1,4-diol (50 c.c.) and conc. hydrochloric acid (2 c.c.). The mixture was occasionally shaken during 3 days. It was then filtered and the filtrate neutralised with solid sodium bicarbonate. After filtration, the volatiles were removed *in vacuo* from the filtrate, leaving a solid that was recrystallised from acetone, then ethyl acetate. The *compound* (**IV**) was obtained as white prisms, m.p. 124–125·5° (Found: N,10·1. $C_{10}H_{18}F_2N_2O_4$ requires N,10·5%).

Ethylenebis-[S-(N-*fluoroacetamido)methyl]dithiocarbamate* (Compound No. 33)

A solution of disodium ethylenebisdithiocarbamate (12·8 g.) in water (80 c.c.) was added, with vigorous stirring, to a solution of *N*-chloromethylfluoroacetamide (from 10·7 g. of *N*-hydroxymethylfluoroacetamide) in acetone (160 c.c.) cooled to −6o°. The temperature rose to −10° and a solid separated. After being kept for 30 min. at room temperature the mixture was filtered off and the solid washed with water. The *compound* (**V**; R = H) was obtained (12·75 g.; 65·4%) as a white amorphous solid, m.p. 180° (decomp.) (Found: N,14·5. $C_{10}H_{16}F_2N_4O_2S_4$ requires N,14·4%). Recrystallisation of a sample (from a mixture of cyclohexanone and ethyl methyl ketone) did not raise the m.p. Some decomposition occurred during the recrystallisation.

Ethylenebis-[S-1-(N-*fluoroacetamido-2,2,2-trichloro)ethyl]dithiocarbamate* (Compound No. 34)

This was prepared in the same manner as Compound No. 33, but with *N*-(1,2,2,2-tetrachloroethyl)fluoroacetamide. The *compound* (**V**; R = CCl_3) was obtained (48·7%) as a white solid (from acetone), m.p. 200° (decomp.) (Found: N,8·9. $C_{12}H_{14}Cl_6F_2N_4O_2S_4$ requires N,9·0%).

N-(2,2-*Dichlorovinyl*)*fluoroacetamide* (Compound No. 35)

A slurry of zinc dust (2·6 g.) in methanol (20 c.c.) was added, with water cooling, to a solution of *N*-(1,2,2,2-tetrachloroethyl)fluoroacetamide (4·7 g.) in methanol (20 c.c.). After 15 min. the mixture was filtered from unreacted zinc. The methanol was removed from the filtrate and to the viscous residue was added water (20 c.c.), then chloroform (20 c.c.). The mixture was shaken well, the organic layer separated, dried (sodium sulphate) and distilled. The *compound* (**VI**) was obtained (1·1 g.; 33%) as a colourless oil, b.p. 91–93°/12 mm.; n_D^{20} 1·5140 (Found: N,7·9. $C_4H_4Cl_2FNO$ requires N,8·1%).

1,3-*Di*(*fluoroacetamido*)-2,2-*di*(*ethoxycarbonyl*)*propane* (Compound No. 36)

Diethyl malonate (32 g.) was added to a solution of sodium (4·6 g.) in ethanol (200 c.c.). The alcohol was removed under reduced pressure and the residual solid was suspended in acetone (200 c.c.). To the stirred suspension was added a solution of *N*-chloromethylfluoroacetamide (prepared from 21·4 g. of *N*-hydroxymethylfluoroacetamide) in acetone (100 c.c.) during 20 min. The mixture was stirred for a further 30 min., then filtered and the filtrate distilled. The *compound* [**III**; R = H, X = $C(CO_2Et)_2$] was distilled as a colourless, very viscous oil, b.p. 180°/0·08 mm.; n_D^{20} 1·4627 (15 g.; 22%) (Found: N,8·0. $C_{13}H_{20}F_2N_2O_6$ requires N,8·2%).

2-(*Dimethyldithiocarbamoylmethoxy*)-1-*fluoroethane* (Compound No. 37)

Paraformaldehyde (11·95 g.) was suspended in a solution of zinc chloride (0·5 g.) in 2-fluoroethanol (25·5 g.). Dry hydrogen chloride was passed into the mixture until all the solid had dissolved (2·5 h.). The reaction mixture then consisted of two layers and was saturated with calcium chloride. The organic (upper) layer was separated from the aqueous layer, dried over calcium chloride and distilled under reduced pressure. 2-Chloromethoxy-1-fluoroethane distilled as a colourless oil, b.p. 86–96°/155 mm. (26·5 g.).

A solution of the crude product (16·9 g.) in acetone (50 c.c.) was added to a hot solution of hydrated sodium dimethyldithiocarbamate (28·2 g.) in acetone (400 c.c.). The mixture was cooled, the precipitated sodium chloride was filtered off, the solvent removed from the filtrate *in vacuo*, the residue extracted with chloroform, the chloroform solution separated from a little water, and then distilled. The *compound* (**VII**) distilled as a colourless oil, b.p. 116–118°/0·3 mm., n_D^{20} 1·5721 (23·4 g.; 72%) (Found: N, 7·0. $C_6H_{12}FNOS_2$ requires N,7·1%).

Experimental and results

Hydrolysis experiments

In order to ascertain the hydrolysis path, certain compounds were treated qualitatively as follows:

N-*Methylenefluoroacetamide derivatives*.—The compound (~50 mg.) was dissolved at room temperature in distilled water (25 c.c.; where solubility was low a saturated solution was prepared). In order to detect the ammonium ion a 0·6% aqueous solution of sodium tetraphenylboron (0·3 c.c.) was added.[5] There was no precipitate. The solution was then heated at 50–55° for 30 min., then cooled. The compounds tested showed no precipitate indicating the absence of the ammonium ion. A saturated aqueous solution of dimedone (1,1-dimethylcyclohexane-3,5-dione; ~5 c.c.) was then added to detect free formaldehyde[6] and the solutions set aside at room temperature for 1 h. *N*-Hydroxymethylfluoroacetamide (Compound No. 1) was twice washed with water before the hydrolysis experiments as the crude material was found to be contaminated with ammonium fluoroacetate. The results of these tests appear in Table III.

N-*Trichloroethylfluoroacetamide derivatives*.—The compound (~50 mg.) was dissolved in ethanol (~20 c.c.), with heating if required. To the cooled solution, distilled water (10 c.c.) was added, followed by a 0·6% aqueous solution of sodium tetraphenylboron (0·3 c.c.). The solution was then heated at 50–55° for 30 min. and cooled. No ammonium ion was detected

Table III

Hydrolysis tests with N-*methylfluoroacetamide derivatives* $FCH_2 \cdot CO \cdot NH \cdot CH_2 \cdot XR$

No. of compound	X	R	Precipitate formed with dimedone
1	O	H	heavy
4	O	Allyl	abundant
6	O	2-Hydroxyethyl	appreciable
10	O	2-(2-Methoxyethoxy)ethyl	heavy
11	O	2-(2-Ethoxyethoxy)ethyl	abundant
12	O	2-(2-n-Butoxyethoxy)ethyl	appreciable
13	O	2-Chloroethyl	slight
14	O	Benzyl	heavy
15	O	2-Acetoxyethyl	appreciable
16	O	2-Acetamidoethyl	none
18	O	Cyclohexyl	none
19	O	1-Methylcyclohexyl	slight
21	O	Benzoyl	none
22	S	*p*-Chlorophenyl	none
23	S	2-Benzothiazolyl	none
24	S	Dimethylthiocarbamoyl	slight
		Name of compound	
26	*N*-(Phthalimidomethyl)fluoroacetamide		none
30	*NN'*-Methylenedifluoroacetamide		none
31	Bis(fluoroacetamidomethyl) ether		heavy
33	Ethylenebis-[*S*-(*N*-fluoroacetamido) methyl]dithiocarbamate		none
	The observed precipitates ranged as follows: heavy > abundant > appreciable > slight		

(a little of compound No. 28 precipitated and was filtered off). In order to detect the presence of free chloral a 0·5% alcoholic solution of 2-hydrazinobenzothiazole was added followed by a 1% aqueous solution of potassium ferricyanide.[7] The solution was kept at room temperature for 1 hour. Chloral under these conditions gave a brown colour. The results of these tests are shown in Table IV.

Table IV

Hydrolysis tests with N-*trichloroethylfluoroacetamide derivatives* $[FCH_2 \cdot CO \cdot NH \cdot CH(CCl_3) \cdot XR]_n$

No. of compound	X	R	*n*	Colour formed with 2-hydrazinobenzothiazole and potassium ferricyanide
27	O	Methyl	1	greenish-yellow
28	S	*p*-Chlorophenyl	1	greenish-yellow
29	S	Dimethylthiocarbamoyl	1	greenish-yellow
34	S	Methylenethiocarbamoyl	2	brown

Formulation

The compounds were formulated as 5% w/v or 20% w/v emulsifiable concentrates in a suitable non-phytotoxic solvent (such as diacetone alcohol) and the emulsifier Lissapol NX (a polyethylene glycol ether), and their activities were tested by various methods.

Contact aphicidal activity

For each treatment three broad bean plants, from which the topmost pair of leaves had been removed (since aphids on these usually closely-folded leaves are difficult to count), were infested with adult and nymphae apterae of *Aphis fabae*. The plants were dipped in the toxicant dilution for 1 sec., fitted with a filter paper collar at pot level in order to prevent the aphids from dropping on to the soil and allowed to dry. Each pot was then placed in a Petri dish filled with water and kept at 75° F and 60% R.H. The assessments were then carried out after 48 h., and the percentage kill calculated, with the correction for natural mortality by Abbot's formula (Tables V–VIII).

Table V

Contact and systemic aphicidal and contact acaricidal activities of N-*fluoroacetamidoalkoxy-compounds*
$FCH_2 \cdot CO \cdot NH \cdot CHR \cdot OR'$

No. of compound	R	R′	Contact activity *Aphis fabae*			Contact activity *T. telarius*			Systemic activity (spraying of broad beans) *Aphis fabae*		
			Kill (%) at p.p.m.								
			100	30	10	1000	250	100	500	250	100
1	H	H	96	67	—*	95	—	—	96	88	—
18	H	Cyclohexyl	100	91	89	87	—	—	100	100	16
19	H	2-Methylcyclohexyl	98	69	13	—	—	—	100	79	—
2	H	Methyl	84	56	—	67	38	32	100	100	95
27	CCl_3	Methyl	6	2	—	88	69	47	38	3	—
14	H	Benzyl	100	87	10	84	—	—	100	100	24
3	H	Ethyl	85	48	21	100	89	86	100	100	96
6	H	2-Hydroxyethyl	83	80	—	98	74	69	87	69	59
7	H	2-Methoxyethyl	96	79	22	98	—	—	100	99	29
8	H	2-Ethoxyethyl	99	64	1	19	—	—	97	93	—
9	H	2-n-Butoxyethyl	98	95	95	4	—	—	100	100	6
10	H	2-(2-Methoxyethoxy)ethyl	92	75	21	89	—	—	98	94	21
11	H	2-(2-Ethoxyethoxy)ethyl	100	50	0	89	—	—	98	78	—
12	H	2-(2-n-Butoxyethoxy)ethyl	95	86	40	25	—	—	99	98	23
13	H	2-Chloroethyl	99	96	91	95	—	—	100	100	—
15	H	2-Acetoxyethyl	100	84	67	10	—	—	99	98	24
16	H	2-Acetamidoethyl	69	41	2	84	—	—	87	8	—
4	H	Allyl	100	100	87	72	—	—	100	99	34
5	H	Prop-2-ynyl	89	81	14	69	—	—	100	93	27
17	H	1,1-Dimethylprop-2-ynyl	—	—	—	15	—	—	—	—	—
21	H	Benzoyl	59	53	—	—	87	78	100	83	—
	Fluoroacetamide		100	98	88	—	—	36	100	100	51

* Not tested at the concentrations marked thus — (Tables V–VIII and X)

Contact acaricidal activity

Each treatment involved two dwarf bean plants, of which the leaflets of the first triad were 5–8 cm. long. A ring of banding grease was placed round the stem below the triad to prevent the mites from moving off the leaves. *Tetranychus telarius* mites were allowed to transfer from pieces of leaf infested with about 50 adult females on to each leaflet of the triad. Each triad was examined to ensure that it bore approximately 150 adults. The plants were then dipped in the toxicant suspension and treated as previously described.

Table VI

Contact and systemic aphicidal and contact acaricidal activities of N-*fluoroacetamidoalkylthio-compounds*
$FCH_2 \cdot CO \cdot NH \cdot CHR \cdot SR'$

No. of compound	R	R′	Contact activity *Aphis fabae*			Contact activity *T. telarius*			Systemic activity (spraying of broad beans) *Aphis fabae*		
			Kill (%) at p.p.m.								
			100	30	10	1000	250	100	500	250	100
20	H	Ethyl	97	93	—	95	—	—	100	100	74
22	H	*p*-Chlorophenyl	98	84	—	99	—	55	99	97	58
23	H	2-Benzothiazolyl	81	—	—	100	—	—	93	90	12
25	H	Cyano	—	—	—	—	89	70	100	100	99
24	H	Dimethylthiocarbamoyl	96	67	47	100	100	97	100	98	—
28	CCl_3	*p*-Chlorophenyl	—	—	—	100	—	—	6	1	—
29	CCl_3	Dimethylthiocarbamoyl	72	22	9	100	—	36	37	3	—

Table VII

Contact and systemic aphicidal and contact acaricidal activities of bis(fluoroacetamide) compounds $[FCH_2{\cdot}CO{\cdot}NH{\cdot}(CHR)_n]X[(CHR)_n{\cdot}NH{\cdot}CO{\cdot}CH_2F]$

No. of compound	R	*n*	X	Contact activity *Aphis fabae*			Contact activity *T. telarius*			Systemic activity (spraying of broad beans) *Aphis fabae*		
				Kill (%) at p.p.m.								
				100	30	10	1000	250	100	500	250	100
30	—	0	Methylene CH_2	99	—	—	90	46	44	100	100	77
31	H	1	O	95	88	—	97	53	32	100	100	85
32	H	1	$O{\cdot}[CH_2]_4{\cdot}O$	100	90	89	97	—	—	100	100	14
33	H	1	Ethylenebisdithiocarbamoyl	82	42	—	—	34	12	75	28	—
34	CCl_3	1	Ethylenebisdithiocarbamoyl	79	45	—	—	99	91	100	97	4

Table VIII

Contact and systemic aphicidal and contact acaricidal activities of various organofluorine compounds

No. of compound	Name of compound	Contact activity *Aphis fabae*			Contact activity *T. telarius*			Systemic activity (spraying of broad bean) *Aphis fabae*		
		Kill (%) at p.p.m.								
		100	30	10	1000	250	100	500	250	100
26	*N*-(Phthalimidomethyl)fluoroacetamide	82	71	—	76	49	43	100	94	4
35	*N*-(2,2-Dichlorovinyl)fluoroacetamide	100	84	15	80	—	—	100	100	48
36	1,3-Di(fluoroacetamido)-2,2-di(ethoxycarbonyl)propane	9	—	—	35	36	22	0	0	—
37	2-(Dimethyldithiocarbamoylmethoxy)-1-fluoroethane*	92	94	15	100	100	97	—	—	—

* The emulsifiable concentrate lost activity on storage at room temperature

Systemic aphicidal activity by spraying broad bean plants

For each treatment three broad bean plants with two pairs of expanded leaflets were used. They were sprayed with the toxicant diluted to 500 and 250 p.p.m. (when activity was high 6 plants were used and sprayed with toxicant diluted to 100 p.p.m.). The shoot tips were protected during spraying with rubber thimbles. Four days after this treatment about 50 aphids (*Aphis fabae*) were allowed to transfer from infested plants to the newly expanded unsprayed leaves and were confined by a ring of banding grease round the stem. Filter paper was placed below these leaves in order to catch the dead aphids. The assessments were carried out 3 days after infestation.

Systemic aphicidal activity by spraying of sugar beet plants

The activity of certain compounds was tested on sugar beet plants at 6–9 leaf stage. The youngest leaf was protected from the spray. Seven days after spraying, about 50 aphids were allowed to transfer on to the unsprayed leaf and isolated there by a ring of banding grease round the petiole. Dead aphids were caught on filter paper. The assessment was carried out 2 days after infestation. (See Table IX.)

Systemic aphicidal activity on broad bean plants by root-uptake from culture solutions

In order to determine the root-uptake and persistence of two compounds, *N*-(1-dimethyldithiocarbamoyl-2,2,2-trichloroethyl,fluoroacetamide (Compound No. 29), a derivative of fluoroacetamide, and 2-(dimethyldithiocarbamoylmethoxy)-1-fluoroethane (Compound No. 37), a derivative of 2-fluoroethanol, were tested. Broad bean plants at 2–3-leaf stage were placed in culture solutions containing the toxicant and infested with *Aphis fabae*. After 48 h. the plants

Table IX

Comparison of systemic aphicidal activity by spraying of sugar beet and of broad bean plants

No. of compound	Name of compound	Systemic activity on *Aphis fabae* by spraying of	
		Sugar beet plants at 500 p.p.m. Kill (%)	Broad bean plants at 100 p.p.m. Kill (%)
—	Fluoroacetamide	65	51
2	*N*-(Methoxymethyl)fluoroacetamide	100	95
18	*N*-(Cyclohexyloxymethyl)fluoroacetamide	29	16
6	*N*-(2-Hydroxyethoxymethyl)fluoroacetamide	99	59
22	*N*-(*p*-Chlorophenylthiomethyl)fluoroacetamide	63	58
30	*NN'*-Methylenedifluoroacetamide	84	77
32	1,4-Bis(fluoroacetamidomethoxy)butane	27	14

were removed and placed in culture solutions free of toxicant. When all the aphids on the plants were killed, the plants were again infested, and re-infestation was repeated until such time as the observed kill was not complete. Compound No. 29 gave complete kill up to 2 days at 25 p.p.m., whereas Compound No. 37 gave complete kill only up to 4 days at 25 p.p.m.

Systemic aphicidal activity by application to stems of broad bean plants

In order to determine stem-uptake and persistence, fluoroacetamide, *N*-(1-dimethyldithiocarbamoyl-2,2,2-trichloroethyl)fluoroacetamide (Compound No. 29), and ethylenebis-[*S*-(*N*-fluoroacetamido)methyl]dithiocarbamate (Compound No. 33) were tested. Four drops of the toxicant at 0·5% concentration were placed at the base of the stem of the bean plants. The plants were infested with *Aphis fabae* 24 h. later and re-infested until such time as the observed kill was not complete.

Fluoroacetamide gave complete kill up to 6 days, Compound No. 29 up to 4 days. The longest complete control was afforded by Compound No. 33, the bis-fluoroacetamide derivative.

Mammalian toxicity of fluoroacetamide derivatives

The acute oral LD_{50} in mice and rats of certain derivatives of fluoroacetamide were determined by Mr. T. R. Middleton of Glaxo Laboratories Ltd.

The compounds were suspended in 0·5% Tween 80 (Compound No. 29) or in 0·2% gum tragacanth (Compound No. 24), dissolved in distilled water (fluoroacetamide and Compounds Nos. 1, 2, 14, 6, 13), or dispersed in 25% propylene glycol (Compound No. 18). The concentrations varied between 4 and 20 mg./ml. Fifty I.C.I. female mice (bodyweight range 19–21 g.) and 30 male Wistar rats (bodyweight range 180–230 g.) were used per determination. The mortality was observed after 7 days (see Table X) except for fluoroacetamide and Compound No. 14 on mice (14 days).

Discussion

Hydrolysis to fluoroacetamide

The contact aphicidal activity of the different fluoroacetamide derivatives varies considerably (Tables V-VII). Poor contact aphicidal activity was shown by *N*-(methoxymethyl)- (Compound No. 2), *N*-(ethoxymethyl)- (Compound No. 3), *N*-(2-acetamidoethoxymethyl)- (Compound No. 16) and *N*-(benzoyloxymethyl)-fluoroacetamide (Compound No. 21) and the trichloromethyl derivatives *N*-(1-methoxy-2,2,2-trichloroethyl)- (Compound No. 27), *N*-(1-dimethyldithiocarbamoyl-2,2,2-trichloroethyl)-fluoroacetamide (Compound No. 29) and ethylenebis-[*S*-1-(*N*-fluoroacetamido-2,2,2-trichloro)ethyl]dithiocarbamate (Compound No. 34).

As, when aqueous solutions of *N*-methylenefluoroacetamide derivatives were heated with sodium tetraphenylboron, there was no ammonium ion present (no precipitate) and formaldehyde was present (demonstrated in a number of cases by the formation of a precipitate with dimedone; Table III), the compounds evidently hydrolyse initially to fluoroacetamide.

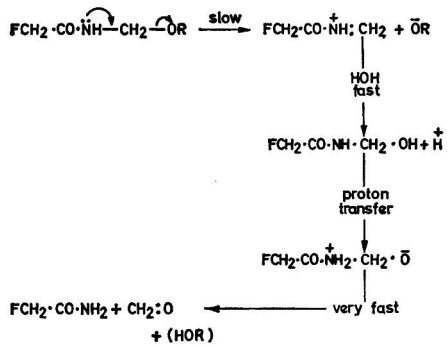
Table X

Mammalian toxicity of fluoroacetamide and certain derivatives

No. of compound	Name of compound	Acute oral LD_{50}, mg./kg. Mice	Rats
	Fluoroacetamide	96*	17
1	*N*-Hydroxymethylfluoroacetamide	160	23
	N-(1-Hydroxy-2,2,2-trichloroethyl)fluoroacetamide	—	60–75**
2	*N*-(Methoxymethyl)fluoroacetamide	136	10–20
18	*N*-(Cyclohexyloxymethyl)fluoroacetamide	100	20–50
14	*N*-(Benzyloxymethyl)fluoroacetamide	180*	33
6	*N*-(2-Hydroxyethoxymethyl)fluoroacetamide	124	16
13	*N*-(2-Chloroethoxymethyl)fluoroacetamide	132	20
4	*N*-(Allyloxymethyl)fluoroacetamide	250	32
24	*N*-(Dimethyldithiocarbamoylmethyl)fluoroacetamide	128	—
29	*N*-(1-Dimethyldithiocarbamoyl-2,2,2-trichloroethyl)fluoroacetamide	830	350

* After 14 days ** Values from B.P. 941,587

Since acetamide[8] and fluoroacetamide[9] are not hydrolysed rapidly *in vivo*, it can be assumed that the first step, in the *in vivo* degradation of the compounds, is also hydrolysis to fluoroacetamide, and then hydrolysis to fluoroacetic acid whose mode of action *in vivo* was discussed by Peters.[10] Indeed, an examination of the systemic activity of the compounds (Tables V–VII) determined after 7 days (cf. 2 days in the case of contact activity) shows it to be similar to that of fluoroacetamide. The compounds would be expected to undergo the hydrolysis shown below.[11] [A similar mechanism would operate for the thio- (Table VI) and bis- (Table VII) compounds.]



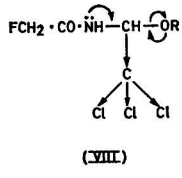
The ease of hydrolysis would depend on the structure and configuration of the compound. Although it is difficult to explain the low contact activities of Compounds 2 and 3, those of the other compounds could be explained on the basis of their increased stability to hydrolysis. The compounds tested for stability showed generally a correlation between their contact activities (Compounds 1, 4, 6, 10, 11, 12, 14, 15, 16, 21, 23, 25, 27, 29, 31, 33, 34) and their ease of hydrolysis (Tables III–VII). Although Compounds 13, 18, 19, 22, 24, 30 showed high *in vitro* stability, their hydrolysis to fluoroacetamide may be facilitated *in vivo* by enzyme activity resulting in their exhibiting high contact activities.

The electron influence of the phenyl ring in Compound No. 21 having the effect of reducing the electron density on the oxygen atom of CH_2-O, and the high electron-affinity of the chlorine atoms in Compounds Nos. 27 (**VIII**), 29 and 34 having the effect of reducing the electron charge on the oxygen atom in $-CH(CCl_3)\cdot O-$, may bring about resistance in these

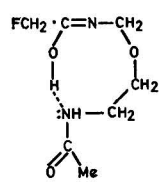
compounds to hydrolysis to fluoroacetamide (Table IV) and a lowering of contact aphicidal activity.

(N.B. The contact activity and lability of the corresponding acetyl derivative—Compound No. 15—were high). Although the longer period employed in the systemic tests allowed the hydrolysis to fluoroacetamide of the more labile compounds (as evidenced by poor contact and good systemic activity of Compounds 2, 3, 21, 23, 24; Tables III–VII), with some compounds (Compounds 16, 27, 28, 29; Tables III–V) resistance to hydrolysis appeared to be considerable, and even after 7 days their systemic activity was low.

The low activity of Compound No. 16 may be due to cyclisation, leading to stabilisation of the compound (**IX**)



(VIII)



(IX)

The contact and systemic activity of 1,3-di(fluoroacetamido)-2,2-di(ethoxycarbonyl)propane (Compound No. 36) is low, probably because of the inability of this sterically hindered compound to penetrate through the cuticle of plant or insect.

That factors other than hydrolytic stability can also operate and exert an effect on the activity of the compounds is shown by several observations. (*a*) Compound No. 29 gave good aphis control by root- and stem-uptake but not by leaf-uptake; (*b*) ethylenebis-[*S*-(*N*-fluoroacetamido)methyl]dithiocarbamate (Compound No. 33) showed poor leaf uptake, but good stem penetration and long persistence, whereas its trichloromethyl-derivative (Compound No. 34) showed poor contact and, surprisingly, good activity by leaf-uptake; (*c*) whereas *N*-(methoxymethyl)fluoroacetamide (Compound No. 2) showed excellent leaf absorption and translocation in bean and sugar beet plants, *N*-(2-hydroxyethoxymethyl)fluoroacetamide (Compound No. 6) was absorbed and translocated more effectively in the sugar beet than in the bean plants.

The systemic activities (leaf-uptake) of the compounds on broad beans at 100 p.p.m. and on sugar beet at 500 p.p.m. were roughly equivalent (Table IX). This may be due to (*a*) better absorption by bean leaves, (*b*) a dilution factor because of the greater weight increase in sugar beet than in bean plants, or (*c*) greater deactivation of the compounds by beet plant enzymes.

The failure of 2-(dimethyldithiocarbamoylmethoxy)-1-fluoroethane (Compound No. 37) to act systemically may be due to the instability of the compound and volatility of its degradation product, 2-fluoroethanol.

Mammalian toxicity

The effect of stability to hydrolysis on the mammalian toxicity of fluoroacetamide derivatives can be seen by comparing the acute oral LD_{50} of *N*-hydroxymethylfluoroacetamide (Compound No. 1) with that of its trichloromethyl-analogue *N*-(1-hydroxy-2,2,2-trichloroethyl)fluoroacetamide, and of *N*-(dimethyldithiocarbamoylmethyl)fluoroacetamide (Compound No. 24) with that of its trichloromethyl-analogue (Compound No. 29) (Table X).

Acaricidal activity

The fluoroacetamide derivatives showed activity against *Tetranychus telarius*, but of a much lower order than against *Aphis fabae*. Trichloromethyl-substitution (Compounds Nos. 27, 34) raised acaricidal activity. Thio-compounds (Nos. 20, 22, 23, 24, 25, 28, 29, 37) were generally more active than oxy-compounds. This is to be expected, since substitution with chlorine and sulphur usually leads to an enhancement of acaricidal activity.

Conclusions

The following tentative conclusions may perhaps be drawn concerning the relationship between activity and chemical structure of *N*-methyl-substituted-fluoroacetamides:

(*a*) insecticidal activity depends on the degradation to fluoroacetamide;
(*b*) greater stability to hydrolysis induced by certain substituents is paralleled by lower activity;
(*c*) the *N*-methylfluoroacetamide derivatives are generally characterised by good systemic activity;
(*d*) the mammalian toxicity of these derivatives is generally lower than that of fluoroacetamide—there is a correlation between stability to hydrolysis and mammalian toxicity;
(*e*) the acaricidal activity of these compounds is enhanced by trichloromethyl- and thio-substitution.

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The Murphy Chemical Co. Ltd.
Wheathampstead
St. Albans, Herts.

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THE YIELDS OF LEAF PROTEIN EXTRACTED BY LARGE-SCALE PROCESSING OF VARIOUS CROPS

By M. BYERS and J. W. STURROCK

The yields of leaf protein that can be expected from the large scale extraction of several crops grown at Rothamsted were determined by measuring the fresh weight of leaves, the dry matter and nitrogen content of the leaf and the percentage of protein-N extracted.

The yields, expressed as kg. of extracted protein per hectare, were greater for cereals than for legumes and other species. The yield depends on species, variety, season, age of the plant and on the ability to regrow after cutting. The response of both wheat and barley to additional fertiliser N was variable. More protein is extractable from young leaves, but in general the percentage of N extracted bears little relation to the N content (as % of dry matter) of the leaf.

The results suggest that by using a suitable succession of crops it should be possible to get 1000 kg. of protein from a hectare in a year.

Introduction

The exploitation of leaves as a source of protein in human nutrition has long been advocated,[1] and in recent years machinery has been developed at Rothamsted and elsewhere to extract protein on a large scale.[2, 3]

This present paper records the calculated yields of extracted protein from various crops, based on the analyses of extracts from bulk processing, and describes how the yield depends on species, variety, the physiological age of the plant and on its regrowth. The effects of generous nitrogen fertilisation with some species are studied.

The crops used were chosen because they are readily available. Many were bred for the production of seed rather than leaf, and there is good reason to expect that other species, including some that are not palatable unprocessed, would give better yields. No immediate large-scale production of leaf protein is however envisaged in Britain, so there is little point in searching for ideal sources here; this research should be done in other climates, especially on leaves that are by-products of crops grown for other purposes. These results are published as a record of what has been done and as an example for future work.

Experimental

Agronomy

Most of the crops were grown on $\frac{1}{4}$ acre plots to provide enough fresh material for bulk extraction. Seed rates were greater than normal to get abundant foliage at early cuts and fertilisers were used more liberally than usual; the cereals, in particular, received large amounts. Trouble from lodging does not arise because the crops are cut while young and still green. Table I shows the seed rates and amounts of fertiliser applied to the various crops. All the cereals, excluding maize, received the same type and quantity of fertiliser, except when the effect of nitrogen manuring on protein extraction was being studied. Details of variations in fertiliser treatment are given at the appropriate place in the text.

Spring cereals are the first crops ready for processing, followed by legumes; the season ends with maize and kale. Cereals, except maize, are first cut when about 8 in. tall; other crops are usually harvested at later stages of growth. Second (or third) harvests taken later are referred to as regrowths, while successive harvests of different parts of the same plot are referred to as age cuts. The area cut and fresh-weight yield were recorded. A modified forage harvester was used which left a stubble about 2–2½ in. high, which cut the crop cleanly and delivered it unbruised into a trailer. Flail-harvested material can be used if it is fed into the pulper within 30 min. but the protein in damaged leaves tends to coagulate and becomes partially unextractable if flail-harvested material has to wait before being processed.

Extraction procedure and sampling

The large-scale extraction of protein from leaves at Rothamsted has already been described.[2, 4a] The machinery continues to be modified but the basic procedure remains the same. The crop is pulped, sometimes with added water, and the fibre left after expressing the first extract is reconstituted with water and re-pressed to give a second extract. The protein is precipitated at 80° by injecting steam into the combined extracts.

A sample of the pulped crop was taken before extraction; if water was used during the first extraction, a sample of the dry pulped leaves was taken first. For a short period, after running conditions had become stable, samples of juice and fibre were collected simultaneously from both the first and second extractions. After being weighed, samples of all fractions were taken for analysis.

Analyses

Pulp and fibre samples were dried to constant weight at 100° and ground to a fine powder in a hammer mill.

A sample of each extract was treated with an equal volume of 10% trichloroacetic acid (TCA) and cooled at +4° for a minimum of 30 min. before centrifuging. The precipitate containing the

Table I

Fertiliser treatment and seed rates of various crops grown for protein extraction

Crop	Year	Fertiliser applied				Seed rate per acre**
		Composition			Rate (cwt./acre)*	
		N	*P*	*K*		
Cereals (except maize)	All years	6	15	15	5·0	12 bu.
		21	0	0	10·0***	
Maize	1961	10	9	9	7·25	75 lb.
	1962	16	9	9	6·3	72 lb.
	1963	20	10	10	6·3	65 lb.
Tares	1959—61	0	12	24	3·0	3 bu.
	1962—64	0	14	28	3·0	3 bu.
Alsike clover and Red clover	All years	0	12	24	3·0	25,40 lb.
Strawberry clover and Sweet clover	1962	0	20	20	4·0	45 lb.
Rape	1962	0	20	20	4·0	33 lb.
	1963	20	10	10	6·0	90 lb.
Mustard	1959—61	16	9	9	10·0	90—120 lb.
	1962	16	9	9	6·0	90 lb.
	1963	20	10	10	5·0	100 lb.
Marrow	1961	16	9	9	7·25	15 lb.
	1962	16	9	9	12·0	45 lb.
	1963	16	9	9	8·0	36 lb.

* British agricultural units: to convert cwt./acre to kg./hectare multiply by 125·5.
** British agricultural units: to convert bushels/acre to kg./hectare for wheat, multuply by 70; rye by 60; barley by 63; tares by 72: to convert lb./acre to kg./hectare multiply by 1·12.
*** Variations of additional N are given in the text.

TCA-insoluble protein-nitrogen (PN) was suspended in a volume of water equal to the volume of the supernatant fluid which contains the non-protein nitrogen (NPN).

The N content of all samples was determined, in duplicate, by microKjeldahl (catalyst: $9K_2SO_4$: $1CuSO_4$:$0{\cdot}02SeO_4$). Total N in an extract is estimated by adding the PN and NPN fractions together. Protein is calculated as N × 6·0.[4b]

Calculation of results

From the weights and N contents of the extracts and fibres the amount of N present in each fraction was calculated. The N content of the ingoing pulp is also known, its weight being taken as the sum of the respective juice and fibre weights. From these figures a N balance sheet was completed for every run, and from it the percent extraction (extractability) of total N and protein N was determined. Both the total N and protein N extracted are expressed as percentages of the total N present in the ingoing pulp. More of the nucleic acid in fresh leaf extracts is precipitated by TCA than by heating to 80°; Singh[5] found that nucleic acid accounted for 6–15% of the N in TCA precipitates depending on the time elapsing between extraction and precipitation, lower values being obtained when the extracts were left for 1 h. or more at room temperature before TCA precipitation.

The amount of protein yielded by a given area is calculated from the weight of foliage, its dry matter and N content, and the protein-N extractability from the leaf. Results are expressed as kg. protein/ha.

Results

Wheat (var. Cappelle)

Winter wheat is harvested in early spring and it grows again extremely well after cutting, allowing two, and sometimes three, harvests.

The crops grown in 1961–1963 received a total of 300 kg. of fertiliser N/ha. Four successive age cuts were made in 1961 and 1962; Table II shows that the yields of both dry matter and

Table II

Performance of wheat (var. Cappelle) 1961–1964

Date of harvest and type of cut			Leaf D.M. %	Leaf N of D.M. %	Extractability, % Total N	Extractability, % Protein N	Yield of D.M., kg./ha.	Yield of protein extracted, kg./ha.
10 May 1961		1 age	14·1	4·36	82·2	71·6	—	—
19		2 age	15·6	3·99	80·9	70·0	2079	348
2 June		3 age	17·7	2·60	84·5	69·6	3623	393
12		4 age	22·9	1·89	64·4	51·7	5824	238
7		1 regrowth	19·2	2·90	72·3	61·0	1924	204
28		2 regrowth	25·4	2·60	69·7	60·8	1053	100
1 May 1962		{1 age}	16·9	4·54	82·3	72·8	1820	359
3			16·2	4·40	73·8	63·0	2000	329
21		2 age	12·9	2·71	65·3	50·7	4510	389
18 June		3 age	23·1	1·58	67·8	50·6	2510	282
21		4 age	23·4	1·73	60·5	44·7	5335	247
24 May		1 regrowth	19·5	2·27	62·6	50·0	2836	192
19 June		2 regrowth	20·3	2·77	60·8	53·3	823	73
22 May 1963		1 age	12·9	5·45	78·9	71·0	919	211
6 June		1 regrowth	16·9	4·42	68·5	58·5	1295	201
1 July		2 regrowth	15·9	3·48	66·3	53·7	1458	163
6 May 1964	Control	1 age	12·4	3·02	74·3	65·4	1702	201
	1N*	1 age	12·6	3·30	68·8	59·0	1813	212
	2N*	1 age	11·6	3·47	71·5	61·2	2250	286
	3N*	1 age	10·4	3·91	72·5	62·2	2481	362
14 May 1964	Control	2 age	14·0	2·50	68·6	58·7	3586	315
	1N	2 age	13·0	2·64	67·7	55·5	3984	350
	2N	2 age	12·9	2·73	66·6	55·0	3625	329
	3N	2 age	10·5	3·28	61·5	47·8	4065	383
28 May 1964	Control	3 age	14·3	1·75	65·5	52·3	5963	333
	1N	3 age	15·2	1·90	71·4	53·3	5514	335
	2N	3 age	15·4	2·01	68·4	52·4	6086	382
	3N	3 age	14·1	2·37	68·0	52·5	5748	420
8 June 1964	Control	4 age	16·3	1·47	59·7	46·6	6870	282
	1N	4 age	15·8	1·50	60·4	47·6	6638	284
	2N	4 age	16·4	1·58	63·5	48·5	6786	311
	3N	4 age	15·7	1·88	63·4	49·1	5476	301
22 June 1964	Control	1 regrowth	16·0	1·18	61·7	50·0	2216	78
	1N	1 regrowth	16·8	1·68	61·2	49·5	3319	166
	2N	1 regrowth	17·6	1·92	67·2	57·0	3116	204
	3N	1 regrowth	16·4	2·22	67·8	57·2	3427	261

D.M. = dry matter

* 1N = 1st Level of nitrogen: 2½ cwt. 21%-N Nitrochalk per acre
2N = 2nd Level of nitrogen: 5 cwt. 21%-N Nitrochalk per acre
3N = 3rd Level of nitrogen: 10 cwt. 21%-N Nitrochalk per acre

extracted protein increase until the end of May; then the latter decreases rapidly, due to the decrease in the extractability of both total N and protein N. Extracts from the first and second regrowths from the first age cut (obtained without further application of N) were similar in composition to those from the later first cuts, although the yields of extracted protein per hectare were smaller. In 1963 the whole crop was cut in mid-May, and regrowths were taken after intervals of 2–3 weeks. The extraction of N from the various harvests followed the pattern of previous years, but the yields of both dry matter and extracted protein did not. Much more foliage was obtained from the regrowths, suggesting that the lighter first cut was less exhausting to the plants than the heavier corresponding ones of 1961 and 1962.

Table III compares the total yields of extracted protein, expressed both as kg./ha. and as a percentage of the first cut, from three successive harvests for the three years. Maximum yields are obtained when the first cut is taken early and can amount to 650 kg./ha.

In 1964 NPK fertiliser was applied at the usual rate, but, to see if the previously used rate of 10 cwt./acre was excessive, top dressings of ' Nitrochalk 21 ' were applied at 2½, 5 and 10 cwt./acre; a control plot received none.

Decreasing the fertiliser N led to an increase in the percentage dry matter and a decrease in

Table III

Yield of extracted protein from successive cuts of wheat, in kg./ha. and as a % of the first cut

	1961		1962		1963	
	Extracted protein, kg./ha.	Yield as %	Extracted protein, kg./ha.	Yield as %	Extracted protein, kg./ha.	Yield as %
1st cut	348	100	359	100	211	100
1st regrowth	204	58·6	192	53·4	201	95·3
2nd regrowth	100	28·7	73	20·2	163	77·3
Total Yield	652		624		575	

the N content of the leaf (Table II), and there was the usual rise in dry matter and fall in N content with increasing age. The extractability of total N and protein N from first cuts was unaffected by variations in treatment, but % extractability was greater from the regrowths on the plots which received 5 or 10 cwt. of ' Nitrochalk '/acre. The response to additional N was greatest at the first of the four age cuts; by the fourth the yields of dry matter and extracted protein increased almost linearly with increasing fertiliser N. Table IV shows the gain in yield at all stages and the efficiency of using extra N (the output of protein N expressed as a percentage of the fertiliser N).

Rye (var. King II)

Table V shows that the yield of protein from the first cuts of rye are comparable with those from wheat. Rye, however, does not regrow well after cutting and quickly runs into ear. The extractabilities are usually less than with wheat, and the ratio of protein N to total N in the extracts is also smaller.

Barley (var. Proctor)

The effects of varied top dressings of N on the yield of extracted protein were studied during 3 years. In 1962 and 1963 ' Nitrochalk 21 ' was applied at 10 and 20 cwt./acre. Only one cut was taken in 1962, but in 1963 two age cuts and the regrowth after the first cut were processed. In 1964 three amounts, 2½, 5 and 10 cwt./acre, were tested; cuts were made at three different ages, and the regrowth after the first cut was also taken. Control plots, receiving no extra N, were grown in all years; all plots received NPK fertiliser at the usual rate.

In 1962 and 1963 the yield of protein was increased fourfold by using 10 cwt. of ' Nitrochalk '/acre; there was no further increase with 20 cwt./acre (Table VI). The yield from the regrowth (20 cwt./acre N only) was approximately three times that of the control.

The experiment in 1964, similar to the one with wheat in that year, was designed to find the amount of N at which the response is maximal, but the results were inconclusive. At the earliest harvest the yield of extracted protein increased with increased fertiliser N, but at the second age cut 5 cwt. of 'Nitrochalk '/acre gave the same yield as 10 cwt. At the third age cut all the plots gave similar yields. These results were similar to those from wheat. However, much less N was taken up than in earlier seasons; with 10 cwt./acre the N content of the leaves increased relative to the control by about 50%, compared with an increase of about 100% in 1962 and 1963. No conclusion can be drawn from the yields of protein from the regrowths. The extractability altered very little over the period of the harvests; only in 1964 was there a slight decline, at the third cut. Except for one result in 1962, fertiliser treatment did not affect the extraction rates.

In 1963, in the 20 days between the first and second cuts, the yields of extracted protein doubled on the fertilised plots, and trebled on the control. The production of dry matter also increased rapidly during this time; most accumulated, at a rate equivalent to 82 metric tons per hectare per year, in the plants receiving 10 cwt. of ' Nitrochalk '. The percentage dry matter of the leaf diminished slightly between these harvests. This fact, coupled with the stable extractabilities, suggests that the increase in dry matter and protein at the second harvest arose mainly from new leaves and not from further accumulation in existing leaves. A similar trend in the dry matter content of the leaves between the first and second cuts was observed in 1964, but the customary increase followed later.

Table IV

Yield of extracted protein and efficiency of use of nitrogen supplied as a top dressing for wheat (1964)

Harvest and treatment	Yield of protein extracted kg./ha.	Gain in yield protein extracted from additional N, kg./ha.	Yield as protein N (protein/6), kg./ha.	N added as fertiliser, kg./ha.	Efficiency in using extra N, %
1st Harvest (6 May)					
Control	201				
1st Level N	212	11	1·8	66	2·7
2nd Level N	286	85	14·2	132	10·8
3rd Level N	362	161	26·8	264	10·2
2nd Harvest (14 May)					
Control	315				
1st Level N	350	35	5·8	66	8·8
2nd Level N	320	5	0·1	132	0·1
3rd Level N	383	68	11·3	264	4·3
3rd Harvest (28 May)					
Control	333				
1st Level N	335	2	0·3	66	0·5
2nd Level N	382	49	8·2	132	6·2
3rd Level N	420	87	14·5	264	5·5
4th Harvest (8 June)					
Control	282				
1st Level N	284	2	0·0	66	0·0
2nd Level N	311	29	4·8	132	3·6
3rd Level N	301	19	3·2	264	1·2
Regrowth (22 June)					
Control	78				
1st Level N	166	88	14·7	66	22·3
2nd Level N	204	126	21·0	132	15·9
3rd Level N	261	183	30·5	264	11·6

Although in 1964 the aggregate of extracted barley protein, from the first cut and its regrowth, on the plot receiving 10 cwt. of ' Nitrochalk '/acre, did not exceed 350 kg./ha., the results of earlier years are comparable with those from wheat. Barley does not regrow as well as wheat, and in 1964 the regrowth was less on some of the plots given additional N than on the control; this accounts for the small yields of both dry matter and protein.

The amount of extra fertiliser N used to produce protein differs from season to season. Table VII shows the percentages of N applied recovered as protein. A peak efficiency of 26%

Table V

Performance of rye (*var. King II*) 1958–1962

Date of harvest and type of cut		Leaf		Extractability, %		Yield of D.M., kg./ha.	Yield of protein extracted, kg./ha.
		D.M. %	N of D.M. %	Total N	Protein N		
9 May 1958	1 age	12·9	3·57	71·8	52·7	3232	294
13	2 age	15·0	3·13	—	—	—	—
27	3 age	18·6	2·13	68·2	51·0	6339	412
9 June	4 age	27·0	1·86	63·1	42·5	8333	394
27 April 1959	1 age	11·8	3·84	79·9	61·3	2444	243
30	2 age	12·6	3·85	74·7	58·6	2534	343
4 May	3 age	13·4	3·16	76·8	57·1	3174	343
11	4 age	15·1	2·82	78·4	50·0	3922	331
21 April 1960	1 age	17·2	3·66	53·5*	46·1*	1616	162*
25	2 age	13·7	3·07	56·0*	52·0*	2336	223*
2 May	3 age	13·1	3·45	75·2	60·2	2645	342
9	4 age	15·3	2·71	47·0	37·7	3104	143
24 May 1961	1 age	16·0	3·61	82·4	61·9	—	—
30	2 age	17·0	2·63	60·5*	46·3*	4459	325*
10 May 1962	1 age	11·2	4·05	65·1	52·0	2597	331

* One extraction only

Table VI

Performance of barley (var. Proctor) 1962, 1963, 1964

Date of harvest and treatment			Leaf		Extractability, %		Yield of D.M., kg./ha.	Yield of protein extracted, kg./ha.
			D.M. %	N of D.M. %	Total N	Protein N		
30 May 1962	Control	1 age	22.5	1·93	49·6	40·8	2013	95
	1N*	1 age	13·3	3·81	64·8	51·4	2962	347
29	2N*	1 age	12·0	4·76	62·8	52·5	2571	383
4 June 1963	Control	1 age	17·8	2·89	68·8	56·2	844	82
	1N	1 age	14·6	4·53	72·7	57·7	1962	301
	2N	1 age	15·5	5·47	65·9	53·5	1672	298
24 June 1963	Control	2 age	17·3	1·65	63·0	51·4	4450	226
	1N	2 age	14·4	2·79	73·3	57·8	6532	633
	2N	2 age	13·0	3·55	66·7	52·8	5187	594
10 July 1963	Control	1 regrowth	21·8	1·38	68·4	53·8	3822	171
	1N	1 regrowth	—	—	—	—	—	—
	2N	1 regrowth	20·8	2·84	68·7	51·3	5289	461
21 May 1964	Control	1 age	14·7	2·55	69·5	59·7	955	77
	1N*	1 age	13·7	2·66	65·1	55·5	1537	136
	2N*	1 age	12·2	2·79	69·9	56·8	1677	159
	3N*	1 age	11·1	3·31	67·5	51·1	1940	197
3 June 1964	Control	2 age	13·8	2·28	70·1	57·0	2723	190
	1N	2 age	13·0	2·33	70·0	57·4	3231	258
	2N	2 age	13·0	2·30	73·9	59·6	3722	306
	3N	2 age	12·8	2·85	71·1	56·6	3003	291
17 June 1964	Control	3 age	18·2	1·54	66·5	52·2	4349	228
	1N	3 age	17·7	1·69	63·3	50·4	3570	182
	2N	3 age	15·6	2·02	63·6	47·6	4113	237
	3N	3 age	16·1	2·34	65·0	50·7	3295	230
23 June 1964	Control	1 regrowth	14·6	2·29	69·7	56·4	1485	114
	1N	1 regrowth	13·8	2·80	70·6	58·8	1028	101
	2N	1 regrowth	13·2	2·85	70·7	59·0	860	86
	3N	1 regrowth	13·0	2·90	72·2	58·7	1379	141

* 1962—63. 1N = 1st Level of nitrogen: 10 cwt. 21%-N Nitrochalk per acre.
2N = 2nd Level of nitrogen: 20 cwt. 21%-N Nitrochalk per acre.
1964. 1N = 1st Level of nitrogen: 2½ cwt. 21%-N Nitrochalk per acre.
2N = 2nd Level of nitrogen: 5 cwt. 21%-N Nitrochalk per acre.
3N = 3rd Level of nitrogen: 10 cwt. 21%-N Nitrochalk per acre.

was reached at the later harvest of 1963. These efficiency values refer only to the N recovered in the extracted protein, and take no account of that left in the fibre residues and in the waste liquors.

Maize (Zea mays)

Three age cuts from four varieties of maize were made over one month in 1961, and over 6 weeks in 1962. In 1963 one variety was studied in more detail. Maize does not regrow well and the regrowth was not harvested. Cobs had reached the silk stage by the late harvests in each year, and they were processed along with the leaves. Less fertiliser N was given than to the other cereals, the total applied per hectare being 145·7 kg. (1961), 126·7 kg. (1962) and 158·4 kg. (1963).

Table VIII shows that in 1961 the yield of extracted protein increased to a peak value about mid-August and then declined; in two varieties it was less at the third harvest than at the first. A single extraction of the leaves was partly responsible for the small yields at the third harvest, but they were also caused by the smaller extractability of protein N. The extractabilities of both total N and protein N did not differ greatly between varieties.

By contrast, in 1962 there was little difference in the yield of extracted protein between the first and second cuts (only one extraction of the latter), but there was a large increase between the second and third because of the increased amount of protein in the crop. As in 1961, there was no varietal difference. Caldera 402 increased in dry-weight yield by nearly 9 metric tons per hectare between the first and third harvests and the yield of extracted protein doubled in this

Table VII

Yield of extracted protein and efficiency of use of nitrogen supplied as a top dressing for barley

Harvest and treatment	Yield of protein extracted, kg./ha.	Gain in yield protein extracted from additional N, kg./ha.	Yield as protein N (protein/6), kg./ha.	N added as fertiliser, kg./ha.	Efficiency in using extra N, %
1962					
1st Harvest (29–30 May)					
Control	95				
1st Level N	347	252	42·0	264	15·9
2nd Level N	383	288	48·0	528	9·1
1963					
1st Harvest (4 June)					
Control	82				
1st Level N	301	219	36·5	264	13·8
2nd Level N	298	215	35·9	528	6·8
2nd Harvest (24 June)					
Control	226				
1st Level N	633	407	67·9	264	25·7
2nd Level N	594	367	61·2	528	10·5
Regrowth (10 July)					
Control	171				
2nd Level N	461	291	48·5	528	9·2
1964					
1st Harvest (21 May)					
Control	77				
1st Level N	136	58	9·7	66	14·7
2nd Level N	159	82	13·6	132	10·3
3rd Level N	197	119	19·9	264	7·5
2nd Harvest (3 June)					
Control	190				
1st Level N	258	68	11·3	66	17·1
2nd Level N	306	115	19·2	132	14·5
3rd Level N	291	100	16·7	264	6·3
3rd Harvest (17 June)					
Control	228				
1st Level N	182	—46		66	
2nd Level N	237	9	1·5	132	1·1
3rd Level N	230	2	0·3	264	0·0
Regrowth (23 June)					
Control	114				
1st Level N	101	—13		66	
2nd Level N	86	—28		132	
3rd Level N	141	26	4·4	264	1·7

time. Pioneer 395 appears to grow faster early in the season; the yields of dry matter and extracted protein exceeded the average for the other varieties at first harvest by 50% and 30% respectively. This variety also gave the best yields at each cut. In 1963 six age cuts were made on Pioneer 395 over a period of 2 months, extending into October. The results again show that yields of dry matter and extracted protein increase with age, although there is no obvious explanation for the mid-season decreases. More nitrogen was extracted this year but this was mainly accounted for by an increase in the NPN content of the extracts.

The value of a second extraction (separate figures not shown, Table VIII gives only the total values) is difficult to assess. In 1961 50% more protein was obtained by a second extraction, but only 10% in 1962. In 1963 40% and 30% was gained at the first and second cuts respectively, and 20% from all remaining harvests. These differences may result from slight alterations in the extraction machinery. However, the total amounts obtained by two extractions of maize were similar in all years. The yield of extracted protein from maize is less than from wheat or barley, possibly because maize plants are more fibrous.

Comparison of the extraction of protein N from cereals

Table IX gives the average extractability of protein N from cereal crops in different years. Results from the fertiliser experiments are included as the use of additional N has little effect on protein extraction. Generally, wheat gives the greatest percentage extraction of protein N.

Table VIII

Performance of maize (Zea mays) 1961, 1962, 1963

Date of harvest, variety and stage of cutting		Leaf		Extractability, %		Yield of D.M., kg./ha.	Yield of protein extracted, kg./ha.
		D.M. %	N of D.M. %	Total N	Protein N		
Caldera 331							
27 July 1961	1 age	12·7	2·64	42·7	31·8	3555	179
16 Aug.	2 age	15·7	1·87	64·7	47·7	8973	480
28	3 age	16·8	1·53	34·0*	24·3*	10181	226*
Caldera 402							
27 July	1 age	16·8	2·72	54·5	45·7	3354	250
16 Aug.	2 age	15·1	1·68	67·3	47·8	8255	352
28	3 age	16·7	1·52	25·8*	18·0*	8589	140*
Orla 266							
27 July	1 age	13·7	2·40	48·8	36·1	4269	221
17 Aug.	2 age	14·1	1·65	65·4	48·5	8068	386
28	3 age	16·8	1·32	31·4*	22·8*	6133	121*
Pioneer 395							
27 July	1 age	14·3	2·81	48·9	37·4	3264	206
17 Aug.	2 age	15·1	1·97	66·6	48·0	6776	380
28	3 age	18·8	1·27	25·6*	18·1*	12126	164*
Caldera 331							
14 Aug. 1962	1 age	13·0	2·23	54·0	37·7	4856	245
4 Sept.	2 age	15·7	1·60	48·4*	36·0*	4847	237*
25	3 age	16·8	1·28	40·9*	27·6*	9188	—
Caldera 402							
14 Aug.	1 age	14·5	1·85	49·2	37·1	4510	187
4 Sept.	2 age	14·7	1·54	42·0*	29·7*	7294	203*
25	3 age	18·3	1·33	56·9	37·2	13338	393
Orla 266							
15 Aug.	1 age	11·3	2·22	55·5	39·1	4187	221
5 Sept.	2 age	11·9	1·43	51·4*	37·9*	5844	191*
26	3 age	17·1	1·29	54·7	35·2	11535	314
Pioneer 395							
15 Aug.	1 age	11·8	2·19	52·6	37·0	5765	283
5 Sept.	2 age	15·1	1·69	47·3*	32·4*	7781	256*
26	3 age	16·8	1·35	55·4	37·9	11560	408
Pioneer 395							
14 Aug. 1963	1 age	12·0	2·65	52·1	33·9	2883	155
21	2 age	11·3	2·52	49·0	30·5	3964	182
30	3 age	11·9	2·36	53·2	39·0	2523	139
11 Sept.	4 age	12·7	2·08	62·2	38·5	2860	144
23	5 age	13·2	1·92	63·1	39·7	4492	204
8 Oct.	6 age	15·4	1·68	59·4	37·6	6743	254

* One extraction only

Legumes

Table X shows the results with various legumes. Age had little effect on the percentage dry matter of any of the crops, but there was the usual decrease in the N content of the foliage with increasing leaf age. The N content of the regrowth was similar to that of the first cut.

The extractability of protein N is less with legumes than with cereals, and only once exceeded 60%. The ratio of protein N to total N in the extract altered little with age. Pea

Table IX

Average extractabilities of protein N for cereals

Year	Maize	Rye	Barley	Wheat
1961	42·9	—	—	62·6
1962	37·7	51·6*	48·8	55·0
1963	36·6	—	54·3	61·1
1964	—	—	55·5	53·5

* 1958—1962 average

Table X

Performance of legumes, 1958–1963

Date of harvest, crop and type of cut			Leaf D.M. %	Leaf N of D.M. %	Extractability, % Total N	Extractability, % Protein N	Yield of D.M., kg./ha.	Yield of protein extracted, kg./ha.
			Red Clover (*Trifolium pratense*)					
1 May 1961		1 age	9·4	3·41	75·7	61·7	2122	267
11		2 age	13·5	2·86	64·5	50·5	—	—
15 June		1 regrowth	16·0	3·24	43·9	38·3	1528	113
20 July		2 regrowth	16·5	3·14	47·8	41·6	1541	120
			Tares (*Vicia sativa*)					
2 July 1959		1 age	18·5	3·90	51·8*	39·4*	1583	146*
8		2 age	23·9	3·56	59·6	42·7	2072	189
17		3 age	17·8	2·93	59·5*	48·6*	2732	233*
23		4 age	18·1	2·37	45·3*	34·0*	3692	178*
29		5 age	16·7	2·52	50·8*	39·6*	4059	242*
30		1 regrowth	17·7	3·55	71·3	56·0	1126	134
3 Aug. 1960		1 age	15·0	3·69	71·1	54·4	3530	425
29 June 1961		1 age	17·3	3·98	54·4	42·6	1959	210
10 Aug.		1 regrowth	23·1	3·15	55·3	41·7	2261	186
13 June 1962		1 age	17·5	5·02	61·7	49·1	981	145
18 July		1 regrowth	21·4	3·48	36·0*	28·0*	2414	111*
29 Aug.		2 regrowth	16·0	3·75	35·5*	28·6*	1499	97*
14 July 1963		1 age	11·5	4·05	51·3*	40·7*	2194	217*
12 Aug.		1 regrowth	16·1	4·22	57·8	44·1	613	68
29 June 1964		1 age	7·8	3·71	61·7	44·6	1975	166
			Sweet clover (*Melilotus alba*)					
18 July 1960		1 age	16·5	2·73	60·9	50·3	3069	252
13 Sept.		1 regrowth	13·7	4·09	64·0	51·6	—	—
24 July 1961		1 age	18·7	1·80	56·7	50·4	1383	74
23 Aug. 1962		1 age	13·0	2·85	48·0*	40·6*	2185	151*
			Strawberry clover (*Trifolium fragiferum*)					
10 Sept. 1962		1 age	14·8	3·50	49·6	40·7	964	82
			Alsike clover (*Trifolium hybridum*)					
22 Aug. 1961		1 age	21·3	3·48	52·2	40·7	1382	117
			White clover (*Trifolium repens*)					
7 July 1958		1 age	11·3	4·26	55·2	37·3	1478	137
14		2 age	12·0	3·75	51·4	36·9	2204	181
16		3 age	11·6	3·15	—	—	—	—
23		4 age	12·5	3·58	—	—	—	—
25		5 age	12·0	3·80	—	—	—	—
31		6 age	12·0	3·59	39·6*	28·9*	2880	171*
18 Aug.	1 regrowth	Age 1	12·3	3·84	—	—	—	—
29	1 regrowth	Age 2	9·6	4·11	—	—	—	—
10 Sept.	1 regrowth	Age 3	11·6	4·01	52·3	36·0	1174	101
24	2 regrowth	Age 1	13·4	3·71	54·5	41·3	1146	105
27	2 regrowth	Age 2	12·2	3·87	—	—	—	—
13 Oct.	2 regrowth	Age 3	10·5	4·06	66·3	55·3	512	69
			Pea haulms (*Pisum sativum*)					
29 July 1958			17·6	2·11	39·2	21·5	—	—
5 Aug.			17·0	2·22	51·5	30·4	—	—
19			14·9	2·29	54·7	27·7	—	—
27			14·1	2·89	67·0	47·0	—	—
2 Sept.			15·4	2·74	54·7	31·6	—	—
11			18·1	3·95	40·7	22·8	—	—
25 June 1959			26·0	1·89	67·3	34·5	—	—
30			18·3	2·24	73·8	45·1	—	—
6 July			21·8	2·14	71·0	36·5	—	—
1 July 1960			22·9	2·39	45·2*	28·3*	—	—
28 Aug. 1962			15·8	2·84	45·9	26·8	—	—

* One extraction only

haulms, obtained as a factory by-product, had the lowest protein-N extractability, sometimes barely exceeding 20%, although the rate for total N remained large. The distance from the factory led to a minimum delay of 3 h. between cutting and processing. The viner in the factory bruises the leaf extensively and this may explain the poor extraction, but we have not tested this experimentally. Table XI summarises the average extractability of protein N for five legumes.

The yield of extracted protein differs greatly from year to year, but tares usually gave good results and in 1960 yielded more than 400 kg./ha. In all years the yield of protein increased with plant age up to the beginning of August.

Forage legumes recover well, providing there is rain after cutting, and satisfactory yields of protein can be obtained from the regrowth. Red and white clovers recover particularly well and can be cut many times. Table XII gives the yield of protein from successive cuts of white clover and tares.

Miscellaneous crops

Protein has been extracted from three members of the Cruciferae, tribe Brassicae, and some other crops; results are in Table XIII. Percentage extraction of both total N and protein N from the cruciferous crops was small, but this was offset by their large fresh weight yields. Six-months-old kale yielded up to 400 kg./ha. of extracted protein, and 6-week-old mustard nearly 200 kg./ha. Although the fresh weight yield of mustard is less than of kale from a corresponding area, it is a useful crop because it grows rapidly. It is practicable to grow three crops on the same piece of land over a 5-month period, thus giving a yield of some 500 kg./ha. extracted protein. Rape gives yields per harvest comparable to mustard, but requires a longer growing period. A regrowth from rape can be obtained when the first harvest is made early, but the other cruciferae tested did not grow well after cutting.

Marrow (*Cucurbita ovifera*) and nasturtium (*Tropaeolum majus*) yield little protein, but both regrow well after cutting when they receive enough water; total yields of extracted protein from marrow can exceed 100 kg./ha. per season.

Sugar beet tops, obtained from several sources gave very variable results probably due to differences in conditions of growth and age at cutting.

Table XI

Overall average extractability of protein N for some legumes

Species	Average extractability of protein N	Number of separate processings on which average is based
Peas (*Pisum sativum*)	32·0	11
White clover (*Trifolium repens*)	39·3	12
Tares (*Vicia sativa*)	42·3	15
Red clover (*Trifolium pratense*)	44·4	5
Sweet clover (*Melilotus alba*)	48·2	4

Table XII

Yield of extracted protein from successive cuts of tares (1962) *and white clover* (1958), *in kg./ha. and as a percentage of the respective first cuts*

	Tares		White clover	
	Extracted protein, kg./ha.	Yield as %	Extracted protein, kg./ha.	Yield as %
1st cut	145	100·0	137	100·0
1st regrowth	111	76·6	101	73·7
2nd regrowth	97	66·9	69	50·4
Total Yield	353		307	

Table XIII

Performance of crucifers, marrow and nasturtium

Date of harvest, crop and type of cut			Leaf D.M. %	Leaf N of D.M. %	Extractability, % Total N	Extractability, % Protein N	Yield of D.M., kg./ha.	Yield of protein extracted, kg./ha.
			Mustard					
22 July 1959		1 age	12·6	3·50	66·9	50·1	1749	184
19 May 1960	Crop 1	1 age	8·4	4·19	17·1*	14·8*	1653	61*
6 July	Crop 2	1 age	13·2	3·63	63·1	49·8	1627	175
20 Sept.	Crop 3	1 age	9·2	3·93	44·5	34·8	—	—
14 June 1961	Crop 1	1 age	9·0	4·02	55·6	41·7	—	—
21 Aug.	Crop 2	1 age	9·3	2·17	66·5	48·7	1034	65
22 Aug. 1962	Crop 2	1 age	9·4	3·57	51·6*	41·9*	2059	182
26 June 1963	Crop 1	1 age	8·8	4·14	41·3	31·4	1225	95
3 July	Crop 2	1 age	7·8	4·07	47·3	36·6	1692	150
			Kale (Successive age harvests)					
1–2 Oct. 1958			11·3	2·75	55·9	34·5	5872	338
15			12·4	2·78	59·2	34·1	—	—
29		Tops	11·1	3·28	60·6	39·0	3289	350
		Stems	15·7	1·86	58·5	24·6	3218	87
27 Aug. 1959			13·4	1·78	63·6	43·1	—	—
10 Sept.			17·9	1·52	62·3	39·9	1190	418
25			21·7	1·07	61·1	46·2	—	—
20 Oct. 1960			12·0	2·25	57·7	36·1	—	—
9 Oct. 1961			13·9	1·67	63·7	49·5	—	—
14–17 Oct. 1963		Pooled sample	12·7	1·97	61·8	36·9	5191	226
			Rape					
4 Sept. 1961		1 age	8·0	3·47	64·4	49·7	—	—
9 Aug. 1962		1 age	9·8	2·53	57·2	38·4	2349	136
19 Sept.		2 age	13·8	2·03	58·2	37·0	3625	161
24 July 1963		1 age	9·1	3·42	58·4	41·6	2145	183
			Marrow					
23 Aug. 1961		1 regrowth	9·8	3·75	59·8	48·1	452	44
28 Sept.		2 regrowth	10·9	3·00	64·3	54·3	113	13
25 July 1962		1 age	9·4	3·87	42·6	35·4	457	38
30 Aug.		1 regrowth	8·9	3·43	36·4	26·7	1447	79
6 Aug. 1963		1 age	9·2	2·93	49·0	37·5	1696	111
			Nasturtium					
21 July 1960		1 age	9·0	3·68	49·5	40·4	1237	110
30 Aug.		1 regrowth	10·5	2·31	23·2*	19·0*	—	—
7 July 1964		1 age	9·7	3·50	56·1	42·2	1159	103

* One extraction only

The by-products of the extraction procedure

It has been emphasised[1] that for the sake of both economy and amenity the by-products should be used. The soluble (NPN) fraction of the steamed extract contains amino-acids, nucleotides and inorganic N, also large amounts of soluble carbohydrate; it could be used as a substrate for growing micro-organisms. The fibre residue can be ensiled or fed direct to animals. Table XIV shows that the N contents of the fibres are usually 1–3% of the dry matter, although with some late-cut cereals it was less than 1% and it exceeded 3% in young plants of some species. The N content of the fibre tends to increase in proportion to the N content of the leaf.

Discussion

The protein yields given here are calculated from N analyses on the various fractions produced during the large-scale processing of leaves. They are therefore ideal figures that would be obtained if it were feasible to conduct the separation without losses during transfer and filtration;

Table XIV

Nitrogen content of the fibre residue from various crops

Date of harvest	and type of cut		N % of D.M. in fibre
WHEAT			
10 May 1961		1 age	2·04
19		2 age	1·48
2 June		3 age	1·54
12		4 age	0·96
7		1 regrowth	1·31
28		2 regrowth	1·46
1 May 1962		1 age	2·47
3		(1 age)	2·35
21		2 age	1·50
18 June		3 age	0·79
21		4 age	0·96
22 May 1963		1 age	2·29
6 June		1 regrowth	2·37
1 July		2 regrowth	2·04
6 May 1964	Control	1 age	1·50
	1N	1 age	1·75
	2N	1 age	1·87
	3N	1 age	2·01
14 May 1964	Control	2 age	1·27
	1N	2 age	1·34
	2N	2 age	1·62
	3N	2 age	1·60
28 May 1964	Control	3 age	0·92
	1N	3 age	0·95
	2N	3 age	1·01
	3N	3 age	1·10
8 June 1964	Control	4 age	0·80
	1N	4 age	0·80
	2N	4 age	0·91
	3N	4 age	0·94
22 June 1964	Control	1 regrowth	0·90
	1N	1 regrowth	0·87
	2N	1 regrowth	0·95
	3N	1 regrowth	1·11
RYE			
27 April 1959		1 age	1·91
30		2 age	1·80
4 May		3 age	1·42
11		4 age	1·19
BARLEY			
30 May 1962	Control		1·44
30	1N		2·34
29	2N		2·98
4 June 1963	Control	1 age	1·86
	1N	1 age	2·77
	2N	1 age	3·33
24 June 1963	Control	2 age	0·96
	1N	2 age	1·30
	2N	2 age	1·50
10 July 1963	Control	1 regrowth	0·92
	2N	1 regrowth	1·44
21 May 1964	Control	1 age	1·50
	1N	1 age	1·55
	2N	1 age	1·59
	3N	1 age	1·95
BARLEY			
3 June 1964	Control	2 age	1·22
	1N	2 age	1·01
	2N	2 age	1·08
	3N	2 age	1·23
17 June 1964	Control	3 age	0·85
	1N	3 age	0·87
	2N	3 age	0·92
	3N	3 age	1·22
23 June 1964	Control	1 regrowth	1·17
	1N	1 regrowth	1·44
	2N	1 regrowth	1·60
	3N	1 regrowth	1·45
MAIZE			
Caldera 331			
27 July 1961		1 age	1·86
16 Aug.		2 age	1·02
28		3 age	1·21*
Caldera 402			
27 July 1961		1 age	2·00
16 Aug.		2 age	0·94
28		3 age	1·12*
Orla 266			
27 July 1961		1 age	1·83
17 Aug.		2 age	1·08
28		3 age	1·41*
Pioneer 395			
27 July 1961		1 age	1·95
17 Aug.		2 age	1·11
28		3 age	1·19*
Caldera 331			
14 Aug. 1962		1 age	1·46
4 Sept.		2 age	1·00
25		3 age	0·96
Caldera 402			
14 Aug. 1962		1 age	1·45
4 Sept.		2 age	1·14
25		3 age	0·98
Orla 266			
15 Aug. 1962		1 age	1·45
5 Sept.		2 age	0·88
26		3 age	0·99
Pioneer 395			
15 Aug. 1962		1 age	1·53
5 Sept.		2 age	1·35
26		3 age	1·08
Pioneer 395			
14 Aug. 1963		1 age	1·65
21		2 age	1·96
30		3 age	1·60
11 Sept.		4 age	1·47
23		5 age	1·17
8 Oct.		6 age	1·12
RED CLOVER			
1 May 1961		1 age	2·07
11		2 age	2·05
15 June		1 regrowth	2·61
20 July		2 regrowth	2·31
TARES			
2 July 1959		1 age	2·69
8		2 age	2·33
17		3 age	1·87*
23		4 age	1·69
29		5 age	1·68
13 June 1962		1 age	3·29
18 July		1 regrowth	2·71
29 Aug.		2 regrowth	3·11
14 July 1963		1 age	3·05
12 Aug.		1 regrowth	3·05
SWEET CLOVER			
18 July 1960		1 age	1·79
13 Sept.		1 regrowth	2·82
STRAWBERRY CLOVER			
10 Sept. 1962		1 age	1·39
ALSIKE CLOVER			
22 Aug. 1961		1 age	2·73
WHITE CLOVER			
7 July 1958		1 age	3·48
14		2 age	3·14
31		(6) age	2·87
PEA HAULM			
19 Aug. 1958			1·74
11 Sept.			3·34
25 June 1959			1·29
30			1·33
6 July			1·49
MUSTARD			
19 May 1960		Crop 1	3·28
6 July		Crop 2	2·33
20 Sept.		Crop 3	2·19
14 June 1961		Crop 1	2·64
21 Aug.		Crop 2	1·99
4 June 1962		Crop 1	2·41
22 Aug.		Crop 2	2·67
26 June 1963		Crop 1	3·12
3 July		Crop 2	3·05
KALE (Successive age harvests)			
1—2 Oct. 1958			1·22
15			1·51
29		Tops	2·16
		Stems	1·30
27 Aug. 1959			0·95
10 Sept.			1·05
25			0·74
RAPE			
9 Aug. 1962		1 age	1·52
19 Sept.		2 age	1·36
MARROW			
25 July 1962		1 age	3·04
30 Aug.		1 regrowth	2·86*
NASTURTIUM			
21 July 1960		1 age	2·13
30 Aug.		1 regrowth	2·07*
7 July 1964		1 age	3·03

* After one extraction only

allowance should also be made for the fact that slightly more N is precipitated by TCA than by heating to 80°. The calculations depend on measurements of the dry matter and N content of the crop and the percentages of total N and protein N extracted. All these vary with changes in the physiological state of the crop, so that the yield of extracted protein is greatly influenced by the conditions of growth and the time of harvest. Different species respond very differently to the same treatment, thus making it impossible to generalise about the optimum time and conditions of harvest; each species needs separate investigation preferably in different climates.

In a study of 28 species growing in Britain, either wild or in a greenhouse, Crook & Holden[6] found that the percentage of N extracted increased as the N content of the leaf increased. Using leaves growing in Ghana, Byers[7] found this relationship to be less precise, and many of our results

are also inconsistent with the earlier observation. The extractability of N from maize, for example, is large in relation to the N content of the leaf; and with barley grown in 1962 and 1963 most N was extracted from those leaves having an intermediate N content (Table VI). Conversely, protein is poorly extracted from many legumes, even when the leaves have a large N content.

The proportion of the total N as protein N in the extracts from all species decreases as the age of the plant increases. Pleshkov & Fowden[8] found that the total N and protein N content of barley leaves (both expressed as a percentage of the dry matter) decreased with increasing age of plant; their figures suggest that the protein N diminishes the more rapidly.

The average amount of protein N extracted is greater from cereals than from the legumes used here (cf. Table IX and Table XI). This poor extraction is not typical of all legumes, because some species grown in Ghana[7] and India[9] extract well.

Cereals (except maize) gave the greatest yield of extracted protein; the yield at first increases with increasing age of wheat and rye, but decreases in early June at the start of flower initiation. The yield from barley can increase considerably during a short period of growth and the decrease starts later than in wheat. These patterns may reflect changes in the uptake of N by the plants, because Watson *et al.*[10] showed that only 70% of the total N absorbed by wheat and barley crops had been taken up by the time the ears emerged. The pattern of change was less consistent with maize, but extractable protein in the variety Pioneer 395 continued to increase until well into the autumn. However, maize is less efficient than wheat and barley, not only because the yield is smaller but also because it is obtained over a much longer period of growth. The yield of legumes increased at least until the beginning of August. Age effects were not studied with the other crops processed, although one result with late-sown rape showed protein increasing up to mid-September.

The optimum time of cutting does not depend solely on yield: for example, Henry[11] found that the protein extracted from older wheat plants had the greater biological value.

Species differ in their ability to regrow after cutting, and the timing of the first cut may have an important bearing on the total yield of extracted protein from a given area; in general, the earlier the first cut, the better is the regrowth. Wheat regrows excellently, barley less well, rye poorly and maize scarcely at all. The yield of extracted protein from regrowth can be considerable (see wheat, Table III). Some of the legumes, in particular red and white clovers and tares, withstand repeated cutting, and in contrast to cereals the N content (as % of dry matter) of their regrowths was similar to that of the first cuts. The decision on harvesting a regrowth depends on all these features.

It is obviously economically advantageous to use as a source of protein, leaves that are by-products of established processes. However, many such are mature and therefore extract less well than the corresponding young foliage; the amount of both total N and protein N extracted from pea haulms and sugar beet tops was extremely variable. The use of waste leaves has particular application in countries which grow a single commercial crop on a large scale.

The maximum output of protein from a unit area of land in a year should be achieved by growing wheat (or barley), cutting one or more regrowths, and following it with a fast-growing crop. Mustard gives a quick return in extracted protein, and two crops taken in succession after mid-summer could add about 400 kg. of extracted protein to the yield from wheat, so that the total yearly production would then exceed 1000 kg. per hectare.

Rothamsted Experimental Station,
Harpenden,
Herts.

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SELF-PROPAGATING DECOMPOSITION IN INORGANIC FERTILISERS CONTAINING AMMONIUM NITRATE

By A. B. PARKER and N. WATCHORN

Certain types of inorganic fertilisers containing ammonium nitrate are capable of self-propagating decomposition if locally initiated by fire or other thermal means and bulk stores have been involved in large scale incidents in several countries. This is a slow type of fuse behaviour new to fertiliser technology and has been entitled ' cigar-burning '. For cigar-burning to occur, an ammonium nitrate and a chloride content are necessary, together with a suitable rigid porous matrix that withstands high temperature. The matrix provides the cellular structure inside which the exothermic chloride-catalysed decomposition of ammonium nitrate takes place. A simple test procedure to differentiate between burning and non-burning compositions is described. Most types of commercially available fertilisers, in particular the important range based on ammonium nitrate, ammonium phosphates and potassium chloride, together with many research formulations have been studied and the effect of formulation on cigar-burning is detailed. Storage recommendations to avoid large scale incidents in the future are given.

Introduction

Inorganic compound fertilisers containing ammonium nitrate have become common in the last decade because of the absence of agronomic problems with ammonium nitrate and its low cost as a source of supplementary nitrogen. The manufacture and storage of considerable tonnages of such fertilisers has not been without incident. Thermal decompositions involving several thousand tons of fertiliser and resulting in appreciable economic loss occurred at Frankfurt-am-Main* in 1961,[1] Cambridge (Maryland, U.S.A.)† in 1963,[2] and Vlaardingen in Holland‡ 1963.[3] Although no explosions or loss of life occurred, the chemical reactions involved created large gas clouds containing toxic elements. These gas clouds have swept across the country side and in once case were tracked by safety and relief organisations on the scale of a national disaster.

Following the first large scale decomposition of an inorganic fertiliser§ in the U.S.A.,[4] Waters *et al.*, explored the character of certain new fertilisers.[5] It is the aim of this paper more clearly to define the fuse-type instability which has caused the major incidents of recent years. Most of the instability of the wholly inorganic fertiliser arises when chlorides are included in the formulation because of their catalytic influence on decomposition of ammonium nitrate. Manufacture with chloride-free materials would appear to be a simple remedy, but it leads to economic difficulties. Potassium chloride is usually employed as the potassic component for economic

* Silo containing 11,000 tons of 12 : 12 : 1 : 2 fertiliser, initiated by fire in underground conveyor
† Stores containing 12,000 tons mainly of 10 : 10 : 10 fertiliser, initiated by building fire of unknown origin
‡ Silo containing 4,000 tons of 12 : 12 : 12 fertiliser, initiation possible from several sources but not defined
§ Silo containing 12 : 12 : 12 fertiliser at Ohio, initiation of undetermined origin

reasons and in any case the frequent presence of chloride in some agricultural grades of potassium sulphate makes chloride difficult to avoid. Problems can, therefore, arise in a wide range of NPK and NK fertilisers.

Compound fertilisers containing ammonium nitrate may show thermal instability in several different ways which are best considered separately since formulations which show one form of instability may or may not be completely inert in other respects. The different types of thermal instability may be classified as:

(*a*) Fuse-type reactions i.e., the passage of a decomposition front throughout a storage pile at ambient temperature after a local thermal initiation.
(*b*) Spontaneous decomposition at high temperatures or 'fume-off '.
(*c*) Self-heating from slow spontaneous reactions in storage.
(*d*) Explosibility from thermal or boostered initiation.
(*e*) Oxidising ability when involved in fire with combustibles.

Thermal instability of type (*a*), which is the subject of this paper, was the cause of the large-scale incidents mentioned above in which large masses of fertiliser stored at ambient temperature decomposed after a relatively minor form of thermal initiation had occurred. Liability to this form of thermal instability is not an inevitable characteristic of fertilisers containing ammonium nitrate and a chloride and it is desirable to avoid this risk by suitable formulation. The test method described later has been used for several years to give a reliable classification of the formulations liable to undergo ' cigar-burning ' (defined below).

In the past cigar-burning behaviour has been confused with type (*b*) instability, i.e., ' fume-off '. ' Fume-off ' is the name[6] given to the decomposition that occurs when the temperature of the whole mass of fertiliser is raised to a high level causing the whole mass to decompose auto-catalytically. This type of decomposition is common to all formulations based on ammonium nitrate and chloride, and results from acidity which is developed spontaneously at high temperatures. ' Fume-off ' is characterised by an induction period whose duration depends on the temperature of the fertiliser mass and the presence or absence of acidic or alkaline components. If inorganic fertilisers which are neutral are kept below 115°, spontaneous development of acidity and ' fume-off ' does not occur. This behaviour is, therefore, only of importance in small storage hoppers etc. in the manufacturing plant where the critical temperature may be exceeded.

Type (*c*) instability, i.e., self-heating, has been described in other publications and mainly concerns fertilisers with both a high free acidity and organic impurities which can be oxidised by the nitrate present. Serious self-heating due to these causes (possibly then resulting in ' fume-off ') can be remedied by adequate neutralisation or addition of urea.[7,8]

Thermal instability of type (*d*) (explosibility) and type (*e*) (oxidising ability) must always be considered when a new fertiliser involving ammonium nitrate is being formulated. These two aspects of thermal instability become important only at very high levels of ammonium nitrate because of the diluent effect of other components. In the general range of NPK fertilisers detonation is unlikely provided contact with conventional explosives is completely avoided.

Self-propagating decomposition (' cigar-burning ')

Fuse-type reactions are a specific form of exothermic decomposition commonly based on an oxidising chemical and a reducing or catalytic agent. They are of interest in the propellant, explosive and allied fields.[9] In the present paper ' cigar-burning ', a term also used in propellant technology, is defined as the decomposition of a fertiliser in a hot, well-defined reaction zone moving through a solid bed at a characteristic velocity. The main research studies of fuse-type reactions (e.g., permanganate–iron or –molybdenum etc.) have been published by Hill[10] and other studies have been reported from Japan.[11] Ammonium nitrate catalysed by chloride was found to propagate too slowly for use as a propellant.[12] Instead, a simple catalysed ammonium nitrate propellant with a small level of chromate catalyst was found to burn much faster and would cigar-burn quite stably at ambient pressure once ignited.[13] Known decomposition catalysts for ammonium nitrate are salts of chromium, cobalt, manganese, nickel and copper, and

in a further group, chlorides and possibly bromides.[14] All these catalysts should be able to induce cigar-burning in some form. However, whereas the first group probably function through an ability to oxidise ammonia,[15] studies by Keenan[16] have shown that the chlorides act in a more complex and less direct manner. Whatever the mechanism, it is not surprising to find that the chloride content of a compound fertiliser may result in an extremely slow form of cigar-burning which hitherto has been little known. This slow cigar-burning has features in common with faster types met in the fuse and delay powder fields.

Cigar-burning of a granular compound fertiliser is illustrated as a laboratory display in Figs. 1 and 2. Two different NPK fertilisers were used both containing ammonium nitrate and chloride. The first figure depicts the localised heating on one end of each tray by a gas burner and the second a subsequent cigar-burning front progressing characteristically in one tray only. The right-hand 22 : 11 : 11* fertiliser (ammonium phosphate type) decomposed locally but was not capable of self-propagating decomposition. It will be shown later that not all fertilisers containing ammonium nitrate and chloride will cigar-burn and that certain ranges of formulations appear to be quite safe in this respect.

Experimental

The standard test procedure

It was necessary to devise a test procedure which would differentiate clearly between burning and non-burning compositions and would be relevant to the storage of fertilisers on a large scale. Sideways propagation in a 25-cm. deep bed was found to be representative of large-scale behaviour and has been used as the standard procedure for classifying new formulations for several years.

The test container is shown in Fig. 3 and requires a 16–18-kg. sample to produce a 25-cm. deep bed. Initiation, by one of the methods detailed below, is carried out in the end zone and any cigar-burning is timed across the major portion of the velocity zone. To time the front movement, three or more iron–constantan thermo-couples protected by simple glass sheaths are inserted to monitor at approximately mid-bed depth at convenient spacings across the velocity zone. Visual following of the surface rate is a useful approximation in most cases but there are exceptions which cannot be monitored in this way. Although it is most important to determine whether a fertiliser is capable of cigar-burning when stored at ambient temperature, a few fertilisers develop burning capabilities only when above a threshold storage temperature. Further tests can, therefore, be carried out on samples preheated up to 100° to indicate any burning potential of a fertiliser during manufacture.

As the name ' cigar-burning ' implies, the mode of initiation does not affect the subsequent burning velocity and as long as stable burning is firmly started over the end face, a variety of alternative methods may be employed including:

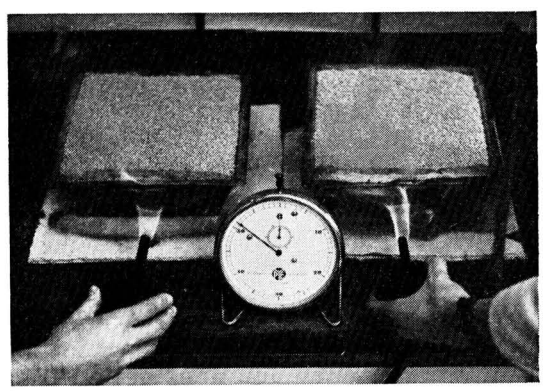

FIG. 1.—*Cigar-burning on laboratory scale—initiation stage*

* Fertiliser analyses are in terms of percentages $N:P_2O_5:K_2O$

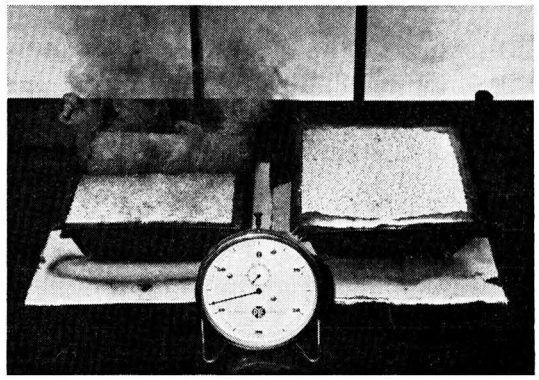

FIG. 2.—*Cigar-burning of NPK fertiliser containing water-insoluble phosphate* (left-hand tray) *and non-burning formulation* (right-hand tray)

(i) Direct external flame heating of the end wall of the initiation zone.

(ii) Buried electric light bulb or electrically heated source.

(iii) Buried sachet(s) of propellant igniter fired from a length of slow burning fuse or by an electric fusehead. Only special slow-burning igniters tend to be successful and moderate success was attained with a 100-g. charge of a type of calcium silicide-copper oxide ignition compound contained in thin film polythene sachet(s). This mixture was relatively slow burning (several seconds) and left a high-temperature slag residue. A safety barricade is always used when this method of initiation is employed.

(iv) Utilisation of a suitable formulation as a primer largely filling the initiation region; the primer is initiated by any convenient method and burning of the sample is then started by the primer burning front. In our work a convenient primer was found to be a 16 : 8 : 25 formulation (monoammonium phosphate type).

(v) Thermal initiation by burying a cluster of steel balls at 800–1000°. A standard procedure uses 1 kg. of Hoffmann $\frac{1}{2}$ in. dia. steel balls at approx. 950° buried just below mid-depth near the wall at the start of the initiating zone.

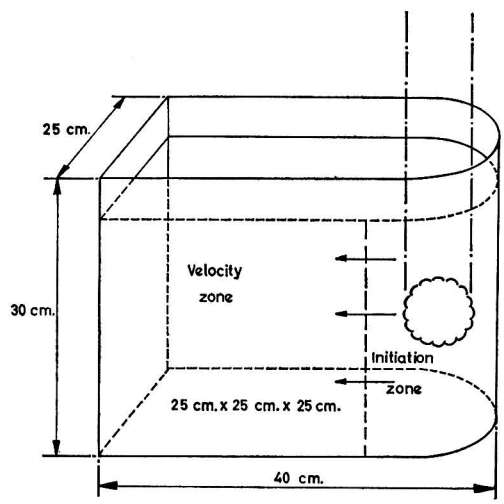

FIG. 3.—*Standard test vessel for cigar-burning ability and front velocity*

In practice, it has been found that method (v) is the most reliable and has been adopted as the standard procedure. Safety precautions have always been taken during the drastic initiation of high nitrate formulations. Formulations containing more than 50% of ammonium nitrate usually flame vigorously when in contact with high-temperature initiators and, although no violence has ever occurred, a sand-bagged barricade is employed as a safety precaution.

The simple rig devised for remote initiation using a metal source is shown in the charging position in Fig. 4. The bulk of fertiliser being tested is placed in the container, a further quantity of fertiliser to fill above the metal source is placed in one of the feed chutes and held in position by a slide that can be remotely operated. Immediately prior to the test, the 1 kg. of $\frac{1}{2}$ in. steel balls preheated to 950° in a muffle furnace, is added and held similarly in the other chute. The operator remotely operates the slides to add the hot metal and the fertiliser respectively and lifts the feed rig out of the test sample using the counter-weight. The main components of the initiation rig are fabricated from stainless steel to resist the corrosive working conditions.

General characteristics of fertiliser cigar-burning

Over 100 fertiliser formulations including commercial products and research materials have been investigated. Details of how the susceptibility to cigar-burning changes with formulation are given later, but certain general characteristics soon emerged which were common to all formulations capable of undergoing cigar-burning.

(1) Formulations which are capable of cigar-burning can be initiated by quite small sources. A single red-hot bolt or buried electric light bulb might initiate burning which could continue throughout a whole silo.

(2) Once initiated, the rate of cigar-burning is independent of the method of initiation and the burning front moves at a velocity characteristic of the formulation.

(3) The liability of a fertiliser to undergo cigar-burning could not be related simply to any single factor such as the ammonium nitrate content. For example, some high-ammonium nitrate fertilisers (50% NH_4NO_3) do not burn, whereas other formulations containing about 25% of NH_4NO_3 can burn at high velocities (e.g., 50 cm./h.).

(4) Although factors such as granule size, the use of pure chemicals etc., can have a minor influence on the rate of burning, they are of secondary importance to the basic chemical composition of the fertilisers.

(5) Two modes of progression of the burning front take place in normal unconfined storage.

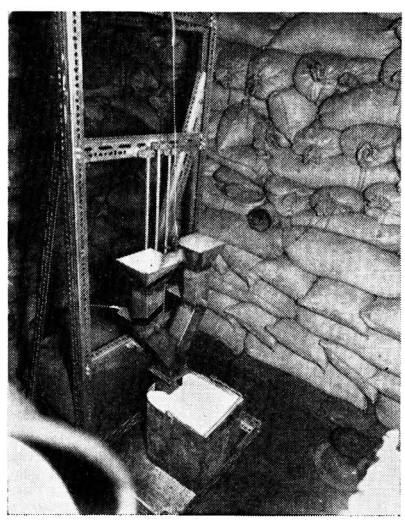

FIG. 4.—*Test procedure: initiation rig in charging position*

The first is the clearly defined cigar-burning which progresses at a characteristic velocity in all directions except the direction in which the decomposition gases escape. The second type of burning occurs in the direction of the escaping gases. In this direction, burning is aided by the hot wet gases and progress is erratic, proceeding by channelling, but is invariably faster than the normal cigar-burning velocity. If initiation occurs in a normal fertiliser pile from a buried source, the direction of gas escape is vertical and, therefore, the whole depth of the fertiliser pile is rapidly involved in the decomposition followed by the vertical face progressing in other directions across the pile at the characteristic cigar-burning velocity.

(6) Complete decomposition of the ammonium nitrate and other volatile materials takes place leaving a residue of the involatile components. The gases evolved vary in composition with the formulation, but in all cases they are toxic because they contain chlorine and its compounds and oxides of nitrogen. These toxic decomposition gases are the chief hazard in any large scale decomposition.

Quantitative studies of initiation requirements

A quantitative study of the thermal requirements for several fertilisers was made, which is pertinent to the use of hot metal in the standard test. Different weights of $\frac{1}{2}$ in. steel balls were preheated to different temperatures and the minimum quantity of metal required at each temperature to initiate burning was determined. Typical requirements were briefly as follows:

Temperature of metal (°C)	350	500	700	800
Successful initiator (kg.)	1—2	0·1—0·5	0·05—0·3	0·03—0·1

It can be seen that although 1–2 kg. of metal at 350° was required (~60 kcal.), substantially less was required as temperature rose and a small number of $\frac{1}{2}$ in. steel balls were effective above 800° (~5 kcal.). It can be seen that the 1 kg. of metal at 950° used in the standard test should adequately initiate all formulations capable of cigar-burning. Occasional tests with ceramic ware instead of steel successfully initiated cigar-burning, showing that no catalytic or other special effects were caused by the use of steel.

Temperature profiles of cigar-burning fronts

The thermal contours of the burning front of a number of fertilisers were determined by recording the temperature as the front passed a stationary thermocouple. The couples were unsheathed iron–constantan approx. 0·5 mm. dia. and the temperatures of the three couples inserted down into the bed were recorded during many experiments. Although the random location of the couples relative to granules and gas space inside the granular bed could produce some irregularity, the experiments revealed that :

(*a*) The thermal wave normally begins with a clearly defined preheated zone caused by conduction and any forward gas penetration. This zone tends to be discoloured and visible due to the effects of temperature and moisture etc. on the fertiliser. Temperatures in this zone do not exceed the boiling point of the fertiliser salt solution (125–135°). Typical widths for this preheated zone are generally 2 cm. but can be as great as 8–10 cm. in some formulations.

(*b*) The main exothermic stage then follows as a rapid temperature rise involving gradients of up to ~ 100°/mm. to a peak which lies in the range 250–480°. The temperature attained at this stage is critical for front movement, as will be shown in the next section.

(*c*) In many fertilisers, this peak reaction temperature is only briefly maintained before the temperature falls away. However, in some formulations the first peak reaction temperature is followed by a slow temperature rise reaching an ultimate maximum 2–3 cm. behind the front before temperatures begin to fall. This second slow temperature rise is particularly noticeable in the case of certain NK fertilisers high in potassium chloride, in which final temperatures as high as 600° have been recorded.

Characteristic cigar-burning temperature profiles for 14 : 14 : 14 (based on citrate-soluble phosphate) and a 16 : 8 : 25 research material (based on ammonium phosphate) are shown in Fig. 5.

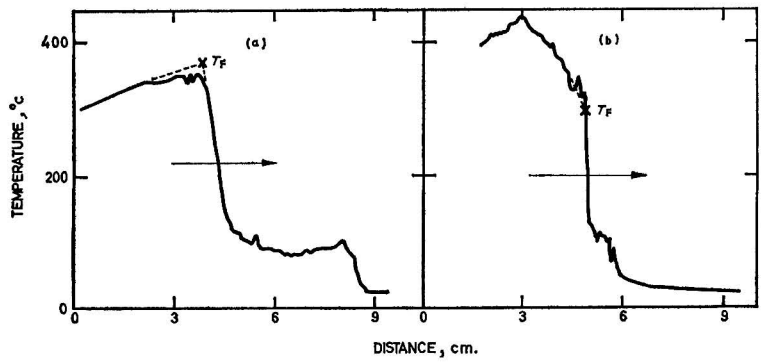

FIG. 5.—*Thermal profiles of cigar-burning reaction zone*

(*a*) 14 : 14 : 14 fertiliser (water insoluble phosphate), German origin (velocity 28 cm./h.)
(*b*) 16 : 8 : 25 research formulation—ammonium phosphate process (velocity 15 cm./h.)
arrow indicates direction of burning
T_F is front temperature as used in Fig. 6

Front temperature and cigar-burning velocity

By determining the temperature profiles of a wide range of fertilisers, it has been shown that a fundamental relationship exists between the characteristic front temperature and the velocity of propagation irrespective of the nature of the formulation. The characteristic front temperature is obtained by extrapolating the cooling or heating curve of the solid residue back to its junction with the main front as shown in Fig. 5. This characteristic front temperature appears to be the peak temperature attained by the solid matrix in the reaction front. The relationship obtained between the characteristic front temperature and the cigar-burning velocity for a wide range of formulations is shown in Fig. 6. One implication from these results is that increases in temperature behind the main front do not exercise any direct control on the normal front movement.

Experimentally, there is considerable evidence to show that ~3–4 cm./h. is the minimum stable velocity and below that formulations do not burn. This finds a ready explanation from Fig. 6 since below 3–4 cm./h. the front temperature would drop below 200° and the decomposition kinetics become too slow to sustain the thermal requirements of the front movement.

Convective effects in front movement

In a porous bed of fertiliser there is considerable scope for gas-phase reactions. The temperature profiles always show a convection zone ahead of the main front and it is important to

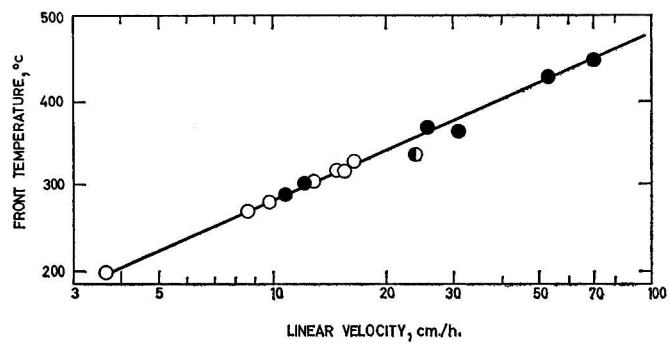

FIG. 6.—*Cigar-burning front temperature vs. linear velocity*

● NPK (water-insoluble calcium phosphate)
○ NPK (ammonium phosphate)
◐ NK (ammonium phosphate–potassium chloride–ammonium sulphate)

know how great a part is played by long range convection and allied gas phase activity. A simple study was, therefore, carried out on the effect of controlling gas flow across the front using a 16 : 8 : 25 fertiliser (ammonium phosphate base, see Fig. 8). For this work the standard test procedure was not used but tests were carried out with a bed 8 cm. dia. × 27 cm. long in a Pyrex pipe. Thermo-couples were inserted to check the effect on the thermal profile. Initiation of burning was carried out with a 100 g. cluster of steel balls at 800° buried at the top of the bed and the burning front was arranged to travel downwards whilst gas flow was controlled or nitrogen was passed either co- or counter-current at different rates. The Pyrex tube allowed a clear view of the burning front, and an experiment in which the front was being purged co-currently, resulting in a deep zone of moist discoloured fertiliser, is shown in Fig. 7. Experimental results are shown in Table I.

By closing the forward vent, the decomposition gases together with additional nitrogen were vented countercurrent to the decomposition front. In this way the overall gas velocity was varied from −150 cm./min. to −205 cm./min. Similarly by closing the rear vent, the decomposition gases and additional nitrogen were vented co-currently and the overall gas velocity varied from +150 cm./min. to +205 cm./min. The results in Table I show that reversing the gas flow changes the rate of movement of the front by a factor of 2 and therefore, in any accidental decomposition, faster propagation in the direction of gas release is to be expected. The 30% variation in gas rate in each direction had little effect on the linear velocity indicating that once burning is established it is relatively insensitive to bulk gas flow.

The minor effects of counter- and co-current gas flow on the delayed reaction zone behind the main decomposition front which occurs with this fertiliser are also suggested in Table I. This tail reaction is therefore probably due to reactions between remnants of potassium nitrate, ammonia etc. trapped in the metaphosphate residue.

Effect of granule size

A detailed study has been made of the effect of granule size on the burning of 16 : 8 : 25 fertiliser (ammonium phosphate base, see Fig. 8). Narrow size cuts of granules in a range from 0·3 to 4·0 mm. together with fines and dust less than 0·4 mm. were obtained by screening and grinding normal product size material, and used in burning tests in a laboratory tray. Slight differences in formulation probably occurred between the fractions. The burning results are shown in Table II.

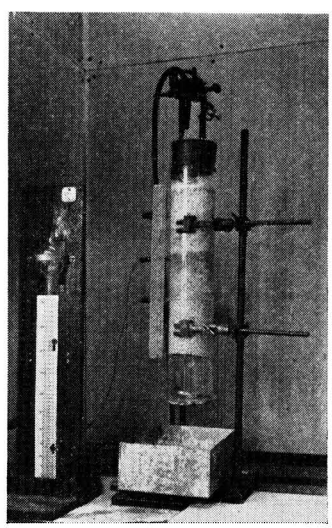

FIG. 7.—*Cigar-burning of* 16 : 8 : 25 *formulation with co-current nitrogen purge*

Table I

Effect of gas flow across burning front

Gas flow control	Front linear velocity, cm./h.	Convection zone depth, cm.	Tail reaction distance to peak temp., cm.	Approx. peak front temp., °c	Tail reaction peak temp., °c
None forward and rear vents open	15·0*	1·75	~3·0**	300	~430**
Countercurrent decomposition gases only*** (forward vent closed)	15·2	0·76	2·0	340	435
Countercurrent N_2 36·7 cm./min.	14·3	0·24	1·2	300	422
Countercurrent N_2 45·7 cm./min.	14·7	0·37	2·0	320	435
Countercurrent N_2 49·4 cm./min.	14·7	0·25	2·0	300	418
Countercurrent N_2 54·8 cm./min.	Failed				
Co-current decomposition gases only*** (rear vent closed)	30·4	7·1	3·1	320	385
Co-current N_2 18·4 cm./min.	32·2	9·1	3·2	280+	464
Co-current N_2 36·7 cm./min.	28·4	4·8	2·4	325	422
Co-current N_2 54·8 cm./min.	33·5	3·3	3·9	280+	405

* Characteristic velocity.
** Thermo-couple failure, typical data from allied experiments.
*** Theoretical maximum evolution at front is 150 c.c./sq.cm.bed/min. based on nitrogen + steam at 350°.

Although the particle size was varied to a considerable degree, the linear- and mass-burning velocities were only moderately altered. A slight trend is shown for velocity to increase as particle size and bed density are reduced. Both these factors tend to occur together, but the results for dust (<0·42 mm.) suggest that it is mainly the bed porosity which is important, although both factors contribute. The highest velocities, therefore, occur in porous beds of dust and in the case of the 16 : 8 : 25 are double the characteristic velocity for normal granules. The later tests on mixtures (Table II) were aimed at high bed densities and devised after several tests had been done to get optimum packing ratios. Although a moderate reduction in bed porosity was achieved, velocities were not significantly affected. The overall results indicate that normal variation in the size analysis of different fertilisers is unlikely to be a significant factor in their cigar-burning behaviour and can be neglected when comparisons of different formulations are being made.

Table II

Effect of granule size on burning of 16 : 8 : 25 *fertiliser*

Granule size (mm.)	Bed treatment	Bed density, g./c.c.	Cigar-burning velocity: Linear, cm./h.	Cigar-burning velocity: Mass rate, g./cm².h.
3·4—4·0	Tapped*	1·01	11·7	11·8
2·4—3·4	Poured	0·92	13·6	12·5
1·68—2·4	Poured	0·93	14·1	13·1
1·20—1·68	Poured	0·95	15·8	15·0
0·85—1·20	Tapped	0·99	12·7	12·6
0·60—0·85	Tapped	1·00	13·5	13·5
0·42—0·85	Poured	0·85	17·5	14·9
0·30—0·42	Mechanically vibrated	0·91	11·4	10·4
Dust (<0·30)	Tapped	0·83	19·4	16·2
Dust (<0·42)	Heavily tapped	1·01	16·9	17·1
Dust (<0·42)	Lightly tapped	0·88	26·4	23·4
Mixtures				
75% (~2·4) 25% (~0·60)	Tapped	1·11	11·39	12·6
75% (~1·68) 25% (~0·42)	Tapped	1·19	13·0	15·5
75% 1·68—2·4 25% 0·21—0·30	Mechanically vibrated	1·31	11·6	15·2

* Settled by manual tapping of container.

Effect of formulation on liability to cigar-burning

A wide range of commercial fertilisers and research formulations has been tested and studied. The fields of NK fertilisers and NPK compounds based on ammonium phosphate have been thoroughly tested and, although the survey of other types of fertilisers is not yet complete, certain general conclusions concerning their liability to cigar-burn are clear. In general, when research formulations were to be tested, conventional manufacturing practice was followed, i.e., wet granulation, drying and screening to a product size of 1·5–3·5 mm., and trade raw materials such as technical grade potassium chloride (60% K_2O) were used. It became clear during the tests that the presence of anti-caking agents on the fertilisers is unimportant and can be neglected. Formulations which were just outside the burning range were often given further initiations by alternative methods such as the primer method [earlier paragraph (iv)] to check whether they were non-burners.

Simple NK formulations (NH_4NO_3 + KCl).—The effect of varying the ratio of ammonium nitrate to potassium chloride on the capability of the material to cigar-burn is shown in Fig. 8 where the compositions lie along the NH_4NO_3–KCl edge of the graph. Compositions showing liability to cigar-burn lie around the stoicheiometric ratio, but burning velocities are slow and hardly exceed marginal rates at any composition. Residue temperatures for those with an excess of KCl however can be particularly high, reaching 500–600°. Wet granulation of NH_4NO_3–KCl mixtures produce a high and often complete degree of interaction to KNO_3–NH_4Cl. There is evidence that the absence of unreacted ammonium nitrate does not restrict cigar-burning and some experiments on NK materials prepared directly from KNO_3 and NH_4Cl did not give significantly different results.

It is now fairly clear that in simple NH_4NO_3–KCl mixtures the K salt has to fulfil two functions. A small amount of chloride provides the necessary catalyst, while the major portion of the K component simply provides a suitable physical solid matrix for burning. The structure provided is not very efficient and none of the simple mixtures of this type, therefore, show vigorous cigar-burning. However, the addition of other apparently inert components can alter the situation radically by providing a more efficient matrix than the excess KCl. The behaviour of NK fertilisers, therefore, deteriorates as certain other components are added and it is in such formulations that the fastest burning is found. For example, a simple non-burning 80% NH_4NO_3–20% KCl mixture to which kieselguhr is added (e.g., making 30% kieselguhr, 55% NH_4NO_3, 15% KCl) becomes a fast burner (linear velocity 150 cm./h.). An excess of other components will eventually act as sufficient ' heat sink ' to make burning impossible but the amount required may be large.

The following sections describe the effect of other fertiliser components on the NH_4NO_3–KCl system.

Ammonium phosphates.—An important class of high-analysis NPK fertilisers is formed by the addition of ammonium phosphate to ammonium nitrate and potassium chloride. In a special study covering a wide range of N : P_2O_5 : K_2O ratios, the ammonium phosphate was restricted to conventional wet process F.D.A.P. analysing approx. 18% N, 48% P_2O_5 (N/P ratio ~1·6). The results of this special study are conveniently summarised in Fig. 8. It can be seen that the formulations capable of undergoing cigar-burning are restricted to a well-defined area which shows a central faster burning zone. In Fig. 9 the commercially important compound fertilisers of this class are shown and it can be seen that few of the commercially important plant-food ratios fall into the burning zone. This limited burning range of the ammonium phosphate-based fertilisers is an inherent safety feature of this class of fertiliser and even in the centre of the burning range shown in Fig. 8 the maximum burning velocity is only ~15 cm./h. The zone of formulations which will burn if stored at ambient temperature is fringed by a narrow belt of materials which will just burn if stored at elevated temperatures, e.g., 85°. With ammonium phosphate-based fertilisers, this belt is narrow and not a major feature. It is only of importance for materials at high temperatures during manufacture, in recycle bunkers etc., or for factory storage when fertiliser is still at elevated temperatures.

Other experiments in which the N/P ratio of the ammonium phosphate varied between 1·0 and 2·0 showed that the N/P ratio does not have an important influence on the liability of a

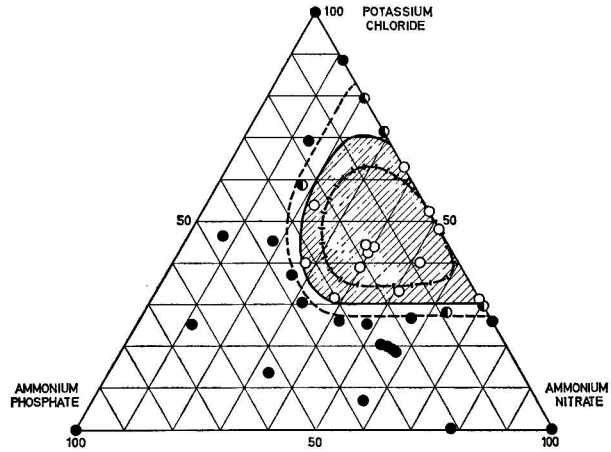

FIG. 8.—*Cigar-burning ability of NPK fertiliser based on ammonium nitrate, potassium chloride and ammonium phosphate (FDAP, N/P 1·6)*

● non-burning ◐ burning ability at 85° ○ burning ability in ambient storage –|–|– velocity contour (6 cm./h.)

fertiliser to cigar-burn. The use of ' Analar ' chemicals instead of fertiliser raw materials for the manufacture of formulations in the centre of the burning zone shown in Fig. 8 resulted in marginally higher rates of cigar-burning.

Ammonium sulphate.—Ammonium sulphate is still a common component of low-grade fertilisers, some of which are enriched by addition of ammonium nitrate. In the samples examined, the addition of ammonium sulphate invariably increased the capability of the fertiliser to cigar-burn. When added on its own to the simple NK fertilisers, the results were quite interesting. The addition of 21% ammonium sulphate to a 67% NH_4NO_3–33% KCl formulation to give a 55% NH_4NO_3, 28% KCl, 17% $(NH_4)_2SO_4$ fertiliser increased the velocity from marginal burning (3–4 cm./h.) to 15 cm./h. Similarly, the addition of 45% ammonium sulphate to a 37% NH_4NO_3–63% KCl formulation increased the velocity from marginal to an erratic high velocity of ~25–90 cm./h. The addition of ammonium sulphate to marginal non-burning formulations in the

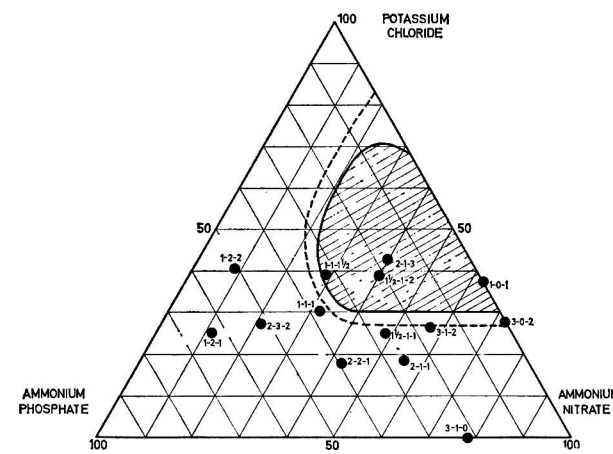

FIG. 9.—*Important NPK fertilisers based on ammonium nitrate, potassium chloride and ammonium phosphate* (FDAP, N/P 1·6)

NPK (ammonium phosphate) class has the effect of increasing the liability to cigar-burn. In practice therefore, dilute fertilisers containing a proportion of ammonium sulphate in the place of ammonium nitrate may be cigar-burners, whereas the concentrated product of the same nutrient ratio containing more nitrate and chloride may not be a cigar-burner. The effect of ammonium sulphate in these compounds may be a result of reacting with any excess KCl to form ammonium chloride and a suitable matrix of K_2SO_4. Its effect when present in fertilisers with many different components is likely to vary.

Other forms of water-soluble phosphate.—Limited evidence available from the study to date suggests that fertilisers made from single and triple superphosphates in water-soluble form may be no worse than those based on ammonium phosphate. The fertilisers available for test contained NH_4NO_3, KCl, $(NH_4)_2SO_4$, single and triple supers. A 14 : 6 : 20 fertiliser corresponding in ratio with the zone of burners in Fig. 8 showed stable burning (~6 cm./h.). A 20 : 10 : 10 blend was a marginal burner and 16 : 9 : 9 and 10 : 10 : 18 fertilisers were marginal burners when at typical recycle bunker temperature (85°).

Citrate-soluble or rock phosphate.—Compound fertilisers containing substantial levels of water-insoluble dicalcium phosphate are marketed in other countries although they are of little importance in the U.K. Two important types of manufacturing processes which result in fertilisers containing a substantial quantity of dicalcium phosphate are the nitro-phosphate processes and the processes based on partial reversion of superphosphates. In processes of the nitro-phosphate type, the rock is acidulated with nitric acid, followed by neutralisation etc. In the other, superphosphates are compounded into the formulation and neutralised. Most of the fertilisers contain potash as KCl. The properties of six commercial fertiliser samples of this type which were available for study are briefly detailed in Table III. The phosphate content was almost all water-insoluble with dicalcium phosphate as the main component.

Table III

Cigar-burning of fertilisers based on water-insoluble phosphate

Fertiliser $N:P_2O_5:K_2O$	Source	% NH_4NO_3	% KCl	Cigar-burning ability (ambient temp.)	cm./h.
14 : 14 : 14	U.S.A.	37	19	Burns	20
16 : 16 : 8	U.S.A.	43	13·5	Burns	25
15 : 15 : 15	Holland	41	24	Burns	~18
13 : 13 : 21	Germany	34	35	Burns	18
12 : 12 : 17 : 2 (Blend*)	Germany	33	15	Burns	58
14 : 14 : 14	Germany	37	27	Burns	~58

* Blend of two fertilisers, one K_2SO_4-based.

All those tested were readily initiated and were in the moderate to fast burning range. The large-scale incidents in U.S.A. and Holland mainly concerned 10 : 10 : 10 and 12 : 12 : 12 fertilisers respectively. Further evidence from the U.S.A.[17] indicates that 15 : 10 : 10, 10 : 20 : 5, and 8 : 16 : 16 fertilisers can all be initiated.

Calcium phosphates present in conventional fertilisers of this type tend to include $Ca_3(PO_4)_2$, $CaHPO_4$ and $Ca(H_2PO_4)_2$ and some ammonium phosphate can be present. The evidence available suggests that the unsatisfactory behaviour is due to the presence of water-insoluble phosphates. Research formulations incorporating rock phosphate had similar properties. Rapid burning was found at phosphate contents as high as 50%. A formulation with main components 31% NH_4NO_3, 7% KCl and 54% rock phosphate had a characteristic velocity of 58 cm./h. Only a small level of chloride was required for the vital catalysis and a similar formulation with only 1% KCl still showed stable burning although with reduced velocity. High KCl contents changed some aspects of behaviour but high velocities were still possible. A fertiliser of this type was unique in showing a marked increase in burning velocity with temperature when above a temperature threshold below which it was non-burning. To confirm that the properties of the insoluble phosphate class were still the effect of chloride, several European fertilisers with very low chloride contents (K present as sulphate) were tested and were non-burners as expected. A

fertiliser free from organic matter (based on calcined rock phosphate) but containing chloride was found to burn at the same high velocity.

Whereas the group of formulations that show cigar-burning is limited in the case of ammonium phosphate fertilisers, there is considerable evidence to show that burning ability will extend over a wide range of fertiliser compositions for fertilisers based on water-insoluble phosphates. The water-insoluble calcium phosphates appear to act as a very efficient matrix for cigar-burning and the chloride content need only be the small level required for catalysis, e.g., 0·5%. Non-burning formulations are likely to be only those of limited commercial importance with low ammonium nitrate contents (15% or less) or extremely high ammonium nitrate contents (70% or more), or fertilisers not based on KCl, or containing substantial levels of specific diluents.

Effect of confinement

The storage piles involved in large-scale incidents have contained up to 12,000 tons of material. Nevertheless, there has been no evidence that the cigar-burning involved in these incidents has altered in any way due to any pressure effects. In all cases where full details were available, the rate of decomposition of the fertiliser has been in accordance with the test results.

In order to check the effect of confinement and to ensure that cigar-burning behaviour could not suddenly change to a more vigorous and hazardous behaviour, an experimental programme has been carried out on the burning of commercial fertilisers and research formulations in vented vessels and bombs. The evidence from these tests shows that it is necessary to restrict the escaping gases by appreciable confinement before different and more vigorous forms of burning begin. These results also show that the actual ammonium nitrate content assumes greater importance when pressurised burning does commence and that the division at ambient pressure between cigar-burners and non-burners does not apply. There is no evidence to suggest that at the confinements likely to be met in large-scale land storage piles the non-cigar-burning formulations would become capable of burning in a self-sustaining fashion.

Discussion

As a result of the work detailed in previous sections, the major factors affecting the cigar-burning of granular fertilisers can be enumerated.

For sustained burning to occur, certain steady-state conditions necessary for the movement of the cigar-burning front must be maintained. The necessary conditions involve physical as well as chemical factors, and formulation changes can completely alter these factors making the maintenance of a self-sustaining steady-state impossible. Such a fertiliser formulation will then only react to a source of heat by undergoing decomposition locally at that source.

Cigar-burning can occur in formulations containing as little as 15% of ammonium nitrate. The chloride ion content required for catalytic purposes can be provided by as little as 1% of potassium chloride. The formulation must also possess a rigid porous structure at high temperature in which the burning can take place. The nature of this high-temperature structure or matrix at the temperatures involved is the most important factor governing burning behaviour. It is thought that the exothermic chloride-catalysed decomposition of ammonium nitrate takes place within the cellular structure provided by the matrix. Whether the cellular geometry or the total surface area is the more important has not yet been determined. The matrix can be provided either by excess potassium chloride, water-insoluble calcium phosphates or specific diluents present in the formulation to the extent of at least 20% and, in some cases, up to 75%.

When excess potassium chloride alone provides the matrix, only slow burning occurs in a limited range of composition. The presence of water-soluble phosphates also only results in a small range of NPK formulations being capable of cigar-burning. Addition of ammonium sulphate or water-insoluble phosphate to an ammonium nitrate-potassium chloride mixture results in more general and rapid rates of burning and a wide range of fertiliser formulations that are capable of cigar-burning. The addition of ' inerts ' can have a similar effect and materials such as kieselguhr provide a very effective matrix resulting in extremely rapid burning.

Although a full discussion on the kinetics involved in cigar-burning is outside the scope of the present paper, it is apparent that:

(*a*) The temperatures of the solid matrix in the reaction zone must be at least 200° before a stable decomposition wave starts to move through the fertiliser.

(*b*) Long-range convective gas effects are not usually important but they do assist in increasing the velocity of movement in a direction co-current with gas discharge.

(*c*) Within normal limits, the sensible heat of the bed is not very important for overall front movement, but raising the bed temperature from ambient to 85° does alter borderline behaviour.

(*d*) An unexpected and close relationship exists between the normal burning velocity and a characteristic reaction front temperature for quite different fertilisers.

Storage

The results provided by this study provide simple but important guidance on storage safety:

(1) Non-cigar-burning formulations, even when containing ammonium nitrate and chloride, can be safely stored in bulk or in bags provided that other safety aspects do not dictate special storage conditions. They may aggravate conditions locally if accidentally involved in a fire but the mass of material should not become involved.

(2) Fertilisers that are capable of fast cigar-burning should not be stored in bulk. The product should be bagged from the plant and stacked with frequent fire breaks in low-fire-risk stores. Workers in all stores likely to handle appreciable quantities should be aware of the fire risk.

(3) It is inadvisable to store even marginal cigar-burning formulations in bulk stores, but if this has to be done, storage should be of fire-proof construction. Underground conveyors etc. should be completely fire-proofed with drencher systems etc. Electrical lighting, heating etc. should be incapable of being buried by the fertiliser. The area should be regulated to reduce fire risks. Sulphur and other combustibles should be avoided.

(4) Although in theory water could be used to deal with cigar-burning, in practice it has met with very little success except in trivial incidents (extremely large fire-fighting teams were quite unable to quell the large-scale occurrences and it proved virtually impossible to penetrate a bulk fertiliser pile using water jets). Provision of adequate water supplies are probably still a necessary precaution when storing some classes of fertiliser. Packaging in plastic bags will minimise spoilage if water has to be used.

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Research & Development Dept.,
Agricultural Division,
Imperial Chemical Industries Ltd.,
Billingham,
Co. Durham

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CONTENTS

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