JOURNAL

OF THE

SCIENCE OF FOOD AND AGRICULTURE

(INCLUDING ABSTRACTS)

Published by the Society of Chemical Industry

Volume 16 | No. 2 | February, 1965

SOCIETY OF CHEMICAL INDUSTRY

FOUNDED IN 1881 INCORPORATED BY ROYAL CHARTER 1907

President:
SIR SYDNEY BARRATT, B.A., LL.D.

Hon. Treasurer:
J. S. GOURLAY, B.Sc., Ph.D.

Hon. Foreign Secretary:
E. L. STREATFIELD, Ph.D., B.Sc., F.R.I.C., M.I.Chem.E.

Hon. Secretary for Home Affairs:
H. K. CAMERON, Ph.D., B.Sc., F.R.I.C., M.I.Chem.E., M.I.E.E.

Hon. Publications Secretary:
PROF. W. G. OVEREND, D.Sc., Ph.D., F.R.I.C.

General Secretary and Editor-in-Chief:
FRANCIS J. GRIFFIN, O.B.E., F.C.C.S., A.L.A.

Editor:
H. S. ROOKE, M.Sc., F.R.I.C.

Advertisement Manager:
P. R. WATSON

Publications Committee:

W. G. Overend (*Chairman*), H. Egan (*Chairman, The Journals and Chemistry & Industry*), G. Brearley (*Chairman, Annual Reports and Monographs*), S. H. Bell, H. J. Bunker, D. V. N. Hardy, B. J. Heywood, J. T. McCombie, S. R. Tailby, W. Wilson, and the Officers

Journals Sub-Committee:

H. Egan (*Chairman*), H. J. Bunker, G. A. Collie, L. C. Dutton, J. Elks, H. Fore, J. K. R. Gasser, J. Grant, J. L. Hewson, T. Jackson, J. H. Nicholas, J. E. Page, A. G. Pollard, J. E. Salmon, M. K. Schwitzer, S. R. Tailby, K. A. Williams, and the Officers

Abstracts Advisory Sub-Committee:

A. C. Monkhouse (Convener), J. N. Ashley, (Miss) D. M. Brasher, H. J. Bunker, C. B. Casson, M. B. Donald, D. Gall, J. E. Garside, A. G. Pollard, and the Officers

Offices of the Society: 14 Belgrave Square, S.W.1

Telephone: BELgravia 3681/5

Annual Subscription to the *Journal of the Science of Food and Agriculture*
£15 post free, single copies £1 17s. 6d. post free

PROTEIN DENATURATION IN FROZEN FISH. IX.*—The Inhibitory Effect of Glycerol in Cod Muscle

By R. M. LOVE and M. K. ELERIAN

The denaturation of cold-stored cod fillets was inhibited to some extent by treatment with glycerol solution before freezing, 10% being the optimum concentration. There was less ice present at a given temperature in frozen tissue pre-treated with glycerol than in the controls, and this observation has shed some light on the nature of the protective mechanism.

Introduction

In early reports[1, 2] and in previous papers in this series[3–5] it was shown that an important factor in the denaturation of frozen cod muscle protein was the increased concentration of the tissue salts resulting from the removal of water as ice. Thus, it was observed that denaturation did not occur in cod at −1·5° if the tissue was merely supercooled, but that it was extremely rapid if the tissue was actually frozen at the same temperature.[5]

It is now well known that the addition of glycerol to suspensions of spermatozoa[6] or blood cells[7] greatly reduces the mortality or haemolysis caused by freezing. The glycerol has been thought[8] to act protectively by lowering the concentration of cell salts in the frozen material, perhaps by causing the tissue water to supercool,[9] since it has itself remarkable ability in this direction.

The aim of the present investigation was to find out whether such a reduction in the salt concentration of frozen cod by glycerol would usefully reduce the extent of denaturation during cold storage. A brief summary of the findings was published[10] in 1962.

Experimental

Materials

Cod (*Gadus morhua*, L.)[11] from 22 to 28 in. long, caught near Aberdeen by trawl net, were used throughout the work. They had been gutted at sea, and stowed in crushed ice for about 3 days before being filleted for use. The glycerol was of B.P. grade, and concentrations have been expressed on a v/v basis unless otherwise stated.

Methods

Denaturation was assessed from the decrease in solubility of the muscle proteins in 5% sodium chloride at chill temperatures, according to the method of Dyer *et al.*[12] essentially as modified by Ironside & Love.[13] Changes in the relative proportions of ice to tissue were observed by measuring the areas of ice and tissue in photomicrographs of histological sections.[10] The concentration of glycerol in tissue samples was determined by the A.O.A.C. method,[14] 0·5–2·0 g. of muscle being first comminuted, without added fluid, in a Marsh–Snow homogeniser[15] for 1 min. at about 9000 r.p.m. No glycerol could be detected in 5 different batches of non-glycerinated cod muscle. 'Free drip' was measured by leaving fillets on steeply sloping trays, studded with metal spikes, at 1° for 24–30 h., and draining off the exudate into measuring cylinders.

Results

Denaturation in cell preparations

A preliminary experiment was carried out with quantities of muscle cells which had been loosened from each other, rather than with whole muscle, in order to obtain as thorough and uniform a penetration of glycerol as possible. Twenty samples of muscle freed from connective tissue,[13] each weighing about 0·5 g., were homogenised in distilled water in a Marsh–Snow homogeniser at about 9000 r.p.m. for 2 sec. Further groups of 20 samples were homogenised similarly in 5, 10 and 15% glycerol. This treatment caused the muscle to separate into individual cells, but did not break the cells further.

* Part VIII: *J. Sci. Fd Agric.*, 1964, **15**, 805

ห้องสมุด กรมวิทยาศาสตร์

The samples were kept in the fluid media for 14 h. at 1°, after which they were filtered on a Buchner funnel and filter paper. Those soaked in glycerol were rinsed with distilled water and the surplus liquid rapidly removed (less than 5 sec.). The cells were then scraped off the filter paper, transferred to small stoppered tubes, frozen overnight in a room at −29° and stored at −14°. Two samples from each treatment were thawed for protein solubility determination every 3 weeks, and the results averaged. The findings are shown in Fig. 1.

It has already been found that denaturation is complete when a value of about 25% solubility has been reached.[16] Fig. 1 shows that soaking in distilled water for 14 h. is sufficient to denature the cod fibrillar proteins almost completely.

In the samples treated with glycerol, it can be seen in spite of the scatter of results that the rate of denaturation was slowest in cells soaked in 10% glycerol, and that it was probably quickest in those soaked in 5% glycerol.

Denaturation in whole muscle

Five pieces of muscle each weighing about 50 g. were cut from the anterior half of each cod fillet, leaving the skin attached. One hundred such pieces were used for each treatment, following which they were wrapped together in aluminium foil in groups of 10 pieces, each from a different fish. They were frozen overnight in still air at −29° and then stored at −14°, one group being removed every 2 weeks for thawing and determination of protein solubility. Controls were frozen without prior soaking.

When the protein solubility/time curves of fish soaked in 5, 10 and 15% glycerol for 4 h. were compared, it was found that the controls and those soaked in 5% glycerol were denatured at about the same rate, but that soaking in 10% and 15% glycerol had reduced the rate appreciably and to a roughly equal extent. When, however, the soaking was carried out for 14 h., there was more discrimination between the different groups (Fig. 2).

It can be seen from Fig. 2 that the samples soaked in 5% glycerol were even more denatured than the unsoaked controls: presumably 5% glycerol was too weak to afford protection to the protein, and the denaturation was caused by the 'water soaking' effect seen in Fig. 1, though to a smaller extent since whole tissue, and not a cell suspension, was involved. It is also clear that 10% glycerol protects the proteins better than 15%.

Table I shows the concentration of glycerol actually present in the muscle samples. After the surface had been quickly rinsed in water, muscle tissue was dissected out, free from connective tissue, to a depth of about 1 cm.—this constituted the 'first layer' of Table I. The

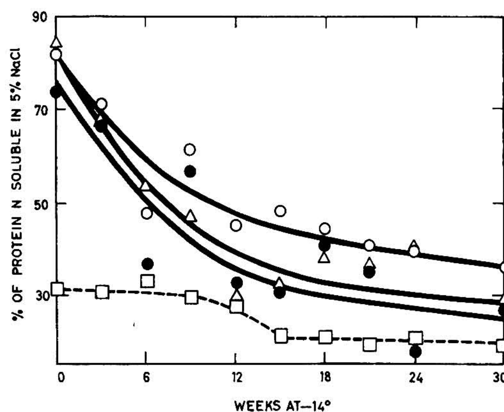

FIG. 1.—*Effect of soaking a suspension of cod muscle cells in glycerol for 14 h. on the solubility of the protein after subsequent storage at −14°*

□ - - - □ Controls, soaked in distilled water for 14 h. before freezing
● —— ● Soaked in 5% glycerol
○ —— ○ Soaked in 10% glycerol
△ —— △ Soaked in 15% glycerol

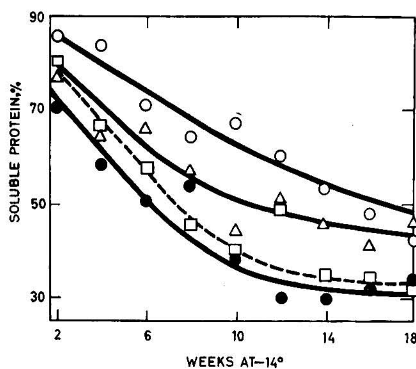

FIG. 2.—*Effect of soaking pieces of cod muscle in glycerol for 14 h. on the solubility of the protein after subsequent storage at* −14°

(legend as Fig. 1 except that controls were not soaked prior to freezing)

Table I

Concentration of glycerol (% *w/w*) *in cod muscle after various treatments*

Strength of glycerol used for immersion	5%		10%		15%	
Period of immersion, h.	4	14	4	14	4	14
First layer	1·69	1·37	2·39	2·76	3·10	3·05
Second layer	0·81	0·99	2·03	2·16	1·65	0·84
Average	1·25	1·18	2·21	2·46	2·38	1·95

The figures are averages from duplicate analyses carried out on pooled samples of muscle from 10 different fish

'second layer' consisted of material taken from the flesh between 1 cm. and 2 cm. from the original surface. The 'second layer' thus reached down close to the skin.

It can be seen that there is no great difference between the average concentrations in muscle soaked in 10% and in 15% glycerol for 4 h., which corresponds with the similarity in denaturation rates already mentioned. However, after 14 h. there is more glycerol in the muscle samples soaked in 10% than in 15% glycerol, and this accords with the denaturation rates illustrated in Fig. 2. The precise mechanism whereby the glycerol concentration in the muscle becomes reduced after increased soaking time in 15% glycerol is not known.

Table I shows that (*a*) soaking in 5% glycerol results in the lowest concentration in the muscle and (*b*) soaking in 15% glycerol results in the greatest amount of glycerol in the surface layer, but that 15% glycerol seems to have poor penetrating power, since the deeper layer contains little more than after a soaking in 5% glycerol.

Influence of freezing rate

Groups of 10 pieces of fillet, each from a different fish as before, were soaked in 7·5% glycerol for 7 h., then wrapped in aluminium foil and frozen (*a*) in still air at −14°, (*b*) in an air-blast at −30° and (*c*) packed in crushed solid carbon dioxide (−78°). It has been shown previously[17] that when cod muscle of approximately this thickness is wrapped in aluminium foil and frozen under these conditions, it is frozen with (*a*) large extracellular ice crystals, (*b*) large intracellular ice crystals and (*c*) small intracellular ice crystals, respectively.

The results (Fig. 3) show no clear difference between the freezing treatments, and suggest that while water will pass through the cell wall in extracellular freezing, some or all of the glycerol remains within the cell in the residual (combined) water and offers protection to the proteins. In intracellular freezing, where the protection is similar, we should therefore probably regard the site of protection as being in the 'protein plus combined water' area rather than the 'ice crystals plus concentrated salts plus free water' area.

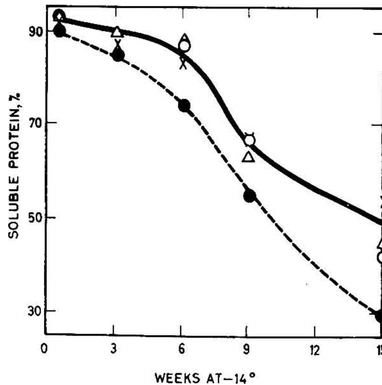

FIG. 3.—*Decrease in protein solubility of pieces of cod muscle during storage at* $-14°$ *after first soaking in 7·5% glycerol for 7 h., and freezing at different rates*

○ Frozen in still air at $-14°$ (large extracellular ice crystals)
× Frozen in air blast at $-30°$ (large intracellular ice crystals)
△ Packed in powdered solid carbon dioxide (small intracellular ice crystals)
● - - ● Control (unsoaked) frozen in air blast at $-30°$

Drip exudation after thawing

Fillets of cod (75) with skins attached and 75 with skins removed were soaked in 10% glycerol for 14 h. at 1°. A further 150 fillets, half with skins removed, were kept as controls without soaking. All fillets were then frozen by placing them, wrapped in aluminium foil, in a room at $-14°$, at which temperature they were subsequently stored. Five fillets were taken for measurements of drip and protein solubility at 3-week intervals.

The drip measurements showed no correlation with denaturation or with the inhibition of denaturation by glycerol. The only clear finding was that the soaked fillets exuded more drip than did the controls: presumably water was imbibed during the soaking.

The relative proportions of ice

Two cubes of tissue with sides 1 cm. long were cut with a fine-toothed saw from the surface near the anterior ends of each fillet which had previously been frozen in still air at -29° and then brought to -14°. Five fillets were used for each treatment, and three contiguous areas were photographed from one section from each cube. In this way an area of about 75 sq. mm. actual muscle was measured in each treatment, embracing about 3000 cells.[10] The percentage area of ice was calculated separately from each photograph. The results are summed up in Table II.

Table II

Area of ice, expressed as % of the total tissue, in fish at $-14°$ *frozen after different treatments*

Treatment	% of ice (area)	Standard deviation	Number of photographs used
None	64	±6·5	27
5% glycerol for 14 h.	65	±6·0	27
10% glycerol for 14 h.	55	±5·5	25
15% glycerol for 4 h.	58	±5·9	31

It can be seen that both 10 and 15% glycerol materially reduce the quantity of ice present, but that 5% glycerol has no effect. The ' control ' figure of 64% is close to previously reported figures[10] of 60 to 62·4%.

Results of taste-panel assessment

Samples were steamed in closed glass casseroles for 30 min. In cod soaked in 10% glycerol for 14 h. it was found in several trials that about half the tasters detected the sweetness imparted by the glycerol, and several found them unacceptable through this cause, although a majority considered the texture to be less tough than in non-glycerinated controls.

However, when 7·5% glycerol for 1 or 2 h. was used, only 1 taster out of a panel of 7 could detect the sweetness, and 6 tasters favoured the glycerinated group as being less tough, after

storage of glycerinated and control groups for 8 weeks at −14°. Similar fillets were given to 10 colleagues to eat at home: two detected the sweetness, but accepted the fish, and the remainder preferred the glycerinated group as being less 'woolly' in texture. A storage trial over 10 weeks at −14° showed that dipping in 7·5% glycerol for 1 or 2 h. before freezing caused the protein solubility to be at least 10% greater than that of the controls, treatment for 2 h. being slightly better than for 1 h.

The improvement in protein solubility brought about by treatment with glycerol therefore reflects an improvement in the texture which is sufficiently large to be detected by tasting.

Discussion

It appears from the present work that 10% glycerol is more effective against denaturation than larger or smaller concentrations. This concentration of glycerol has also proved to be optimal in the hands of others for preserving viability in frog spermatozoa,[18] rat liver mitochondria,[19] canine blood platelets[20] and shigella (a pathogenic bacterium).[21] Spermatozoa have also been preserved in 15% glycerol in normal saline,[22] and it has been shown[8] that the concentration of glycerol required to prevent the rupture of red blood cells during freezing depends on the temperature to be used: 5·4% prevents it at −10°, but 24% is needed at −40°, to quote the extreme values.

Relatively high concentrations of glycerol have been found naturally in a species of insect larva, which enable it to remain supercooled during severe winter conditions.[23] Glycerol is not, however, the 'anti-freeze' agent in fishes found in the Arctic, which also supercool for long periods.[24]

Merely soaking cod cells in water for 14 h. (Fig. 1) was sufficient to cause denaturation, illustrating once more the extraordinary lability of cod actomyosin. A hint of this phenomenon has been reported earlier:[25] small pieces of cod muscle left for several hours in a dish cooled with ice showed a slight development of denaturation, as measured by the cell fragility method,[25] apparently because atmospheric water condensed on to the samples. In each case the ionic environment of the actomyosin was altered, causing a sort of irreversible precipitation *in situ*. It is a matter of common experience that when actomyosin is precipitated out of a salt solution by dilution with water, it will not all redissolve in the same solvent; some of the protein has become damaged during precipitation. It would seem that an analogous, though more extreme, situation has developed in the present work.

Glycerol reduces the proportion of ice present in the tissue. Luyet & Gehenio[9] estimated calorimetrically the weight of beef muscle converted to ice at −30°, and found it to be 73% in untreated controls and 54, 41 and 28% in tissue pre-treated for 10 min. with 30, 60 and 100% glycerol, respectively. The present histological studies show (Table II) that the same effect occurs in cod, and in addition they show that the glycerol acts in the 'protein plus combined water' phase: if it had merely increased the amount of unfrozen free water, then the 'ice plus free water' areas in the photographs would have remained the same as in the controls, since any liquid water and glycerol would be washed out during the histological preparation and appear finally in the photographs as ice. If it be accepted that, as outlined in the Introduction, denaturation is caused directly or indirectly by the concentration of tissue salts by freezing, then it seems likely that the glycerol protects the protein by increasing the relative proportion of the 'protein plus combined water' phase, diluting the salts in it, and not by reducing the damaging effect of any 'extra-protein' pockets of liquid salt solution that there may be.[4]

Some recent work[26] has revealed a new aspect of protein denaturation in frozen cod, which allows the possibility of a different interpretation of these results. Evidence showed that cod-muscle protein bound water with varying degrees of affinity. Removal of the more tightly bound water as ice by freezing to steadily lower temperatures resulted in a progressively increasing damage to the protein.

It is possible, therefore, that glycerol inhibits denaturation at low temperatures by the very fact of maintaining more water in association with the protein (Table II), rather than by dilution of the inorganic ions.

The toxicity of glycerol is very low—the human body carries at all times about 1% of glycerol under conditions of dynamic equilibrium—and it can be absorbed and metabolised without difficulty in amounts of up to 50% of the normal diet.[27] It has also been calculated that the average adult would have to ingest some 480 g. of glycerol before the minimum symptomatic level is reached.[27] It may be felt that the protection afforded to the protein of cod fillets by glycerol solutions is insufficient to justify its use in industry, but reference to denaturation curves in cod at different temperatures[4] shows that fillets soaked in 10% glycerol for 14 h. and stored at −14° are denatured at a rate comparable with non-glycerinated controls stored at about −20°. The advantage of being able to use a higher storage temperature in a hot country is obvious. The cost of the glycerol would not exceed 2d. for every 10 lb. of fillets.

Acknowledgment

The work described in this paper was carried out as part of the programme of the Department of Scientific and Industrial Research. One of the authors (M. K. E.) was in receipt of a grant from the United Arab Republic.

Torry Research Station
Aberdeen

Received 19 June, 1964

References

[1] Reay, G. A., *Rep. Fd Invest. Bd*, 1930, p. 128 (London: H.M.S.O.)
[2] Notevarp, O., & Heen, E., *Fiskeridirectoratets Shrift. Ser. Teknol. Undersøk.*, 1938, **1**, No. 2
[3] Love, R. M., *J. Sci. Fd Agric.*, 1958, **9**, 609
[4] Love, R. M., *J. Sci. Fd Agric.*, 1962, **13**, 269
[5] Love, R. M., & Elerian, M. K., *J. Sci. Fd Agric.*, 1964, **15**, 805
[6] Polge, C., Smith, A. U., & Parkes, A. S., *Nature, Lond.*, 1949, **164**, 666
[7] Smith, A. U., *Lancet*, 1950, ii, 910
[8] Lovelock, J. E., *Biochem. J.*, 1954, **56**, 265
[9] Luyet, B. J., & Gehenio, P. M., *Biodynamica*, 1952, **7**, 107
[10] Love, R. M., *J. Fd Sci.*, 1962, **27**, 544
[11] Cohen, D. M., *J. Cons. int. Explor. Mer.*, 1959, **25**, 50
[12] Dyer, W. J., French, H. V., & Snow, J. M., *J. Fish. Res. Bd Can.*, 1950, **7**, 585
[13] Ironside, J. I. M., & Love, R. M., *J. Sci. Fd Agric.*, 1958, **9**, 597
[14] 'Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists (A.O.A.C.)', 9th edn, 1960, p. 225 (Washington, D.C.: The Association)
[15] Marsh, B. B., & Snow, A., *J. Sci. Fd Agric.*, 1950, **1**, 190
[16] Love, R. M., & Ironside, J. I. M., *J. Sci. Fd Agric.*, 1958, **9**, 604
[17] Love, R. M., *J. Sci. Fd Agric.*, 1955, **6**, 30
[18] Rostand, J., *C. R. Acad. Sci. Paris*, 1946, **222**, 1524
[19] Greiff, D., Myers, M., & Privitera, C. A., *Biochim. biophys. Acta*, 1961, **50**, 233
[20] Cohen, P., & Gardner, F. H., *J. clin. Invest.*, 1962, **41**, 10
[21] Nakamura, M., Farnum, J. L., & Oke, M. A., *Nature, Lond.*, 1962, **194**, 405
[22] Parkes, A. S., *Proc. III int. Congr. Animal Reproduction* (Cambridge), 1956, 7 pp.
[23] Salt, R. W., *Nature, Lond.*, 1958, **181**, 1281
[24] Gordon, M. S., Amdur, B. H., & Scholander, P. F., *Biol. Bull., Woods Hole*, 1962, **122**, 52
[25] Love, R. M., & Mackay, E. M., *J. Sci. Fd Agric.*, 1962, **13**, 200
[26] Love, R. M., & Elerian, M. K., *C. R. XI Congr. int. Froid*, 1963, paper 4.1
[27] Newman, A. A., *Food Manuf.*, 1964, **39**, 36

DETERMINATION OF THE WATER ACTIVITY OF SOME HYGROSCOPIC FOOD MATERIALS BY A DEW-POINT METHOD

By G. AYERST

A dew-point apparatus for the determination of the water activity of stored foods and other substances is described and its precision assessed. The results of measurements at two temperatures and over a range of moisture content on a number of varieties of foods are presented ; they are discussed in relation to the phenomenon of hysteresis and used to test two published equations relating water activity to moisture content and temperature.

Introduction

When water is absorbed by a hygroscopic material or has substances dissolved in it, its vapour pressure (P), stated as a fraction of the vapour pressure of pure water at the same temperature (P_0), is a measure of its availability for biological processes including the growth of specific arthropods and micro-organisms.[1-3]

In this paper the term water activity a_w[1] will be used for P/P_0 when referring to the water in the product and the term percentage relative humidity ($100\,P/P_0$) for the status of water in air.

The problem of determining the water activity of a moist hygroscopic material resolves itself into finding the atmospheric relative humidity at which the material neither gains nor loses moisture. In the graphical interpolation method,[4] replicate samples of material of known moisture content are exposed to atmospheres of different relative humidity and the relative humidity at which no weight change occurs is found by interpolation. In the isopiestic method,[5] samples of the material under investigation are held in a vacuum thermostat together with a solution of a salt for which the relationship between water activity and concentration is known. At equilibrium the water activity of the material is defined by the concentration of the reference electrolyte. This method is particularly valuable for the measurement of very high water activities.

In most other methods,[4] either the material is allowed to change in moisture content until it is in equilibrium with an atmosphere of known relative humidity, or a small volume of air is allowed to come into moisture equilibrium with a sample of material and its relative humidity is then measured. The main advantage of this latter group of methods is that, because the water content of air is relatively small, equilibrium is attained quite rapidly. The main difficulty is in the accurate measurement of the relative humidity of small volumes of air, but several workers have used vapour pressure measurements,[4] dew-point techniques[6, 7] and electrical hygrometers[8, 9] to do this.

For the present investigation a dew-point apparatus was designed to reduce some of the errors inherent in the method. This technique was chosen because relatively large samples of material can be tested, it is rarely affected by the presence of volatile substances other than water, measurements can easily be made on the same sample at more than one temperature, and the only calibration required is that of the temperature-measuring device.

The apparatus has been used to measure the relative humidity of air in equilibrium with samples of several hygroscopic foods and these measurements have been related to the moisture contents of the samples, determined by standard commercial laboratory methods. It has also been used successfully to check the water activities of fungal culture media and of humidity-controlling solutions.

Water sorption isotherms of most of the materials studied on this occasion have been published previously, for example wheat,[9-12], coffee,[13] sorghum[12, 14] black seed pepper[15] and groundnuts[16, 17] but many of these measurements were made only at one temperature or only at water activities below about 0·8.

Experimental

Measurement of equilibrium relative humidity

The apparatus (Fig. 1) is contained in a rectangular water bath of about 20 l. capacity,

the temperature of which is regulated by a mercury contact-thermometer controlling a 300-W heater through an electronic relay. The bath is cooled by a copper coil through which tap water or other coolant may be passed and is stirred by a centrifugal pump.

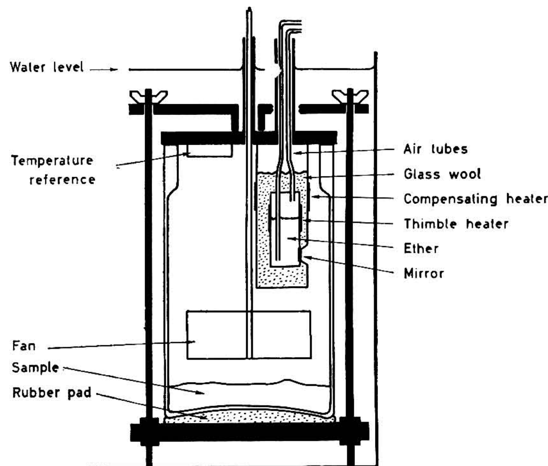

FIG. 1.—*The dew-point apparatus*

The wide-mouthed, 3-l. glass jar in which observations are made rests on a soft rubber pad on a heavy gun-metal base. The machined gun-metal lid is clamped in place by a brass bar with a central collar and the metal-to-glass joint is sealed with high-vacuum grease. The weight of metal is sufficient to ensure that the jar is stable when it is submerged in the water bath.

This jar contains the material to be examined, the dew-point thimble and a low-speed wide-bladed fan. Three 36 s.w.g. copper/constantan triple thermopiles are used for measuring temperatures with reference to a copper block which is screwed to the lid. The distant junction of the first of these thermopiles is suspended about halfway between the thimble and the wall of the jar in order to measure the temperature of the air in the jar. The second, which is covered by a polythene tube, is immersed in the water bath and the third is used to measure the temperature of the dew-point mirror.

The 20-ml. dew-point thimble is constructed of copper with the mirror (a 3-mm.-thick block of silver with an exposed highly polished surface of about 0·7 $cm.^2$) soldered over a hole near its base. It is cooled by bubbling air through light petroleum which is poured in through the air inlet tube. The three junctions of the triple thermopile are inserted into evenly spaced 1-mm. holes in the silver parallel to the mirror face, and are held in place and electrically insulated from the silver by epoxy-resin. A 0·3-W to 1·2-W variable heater is wound on the thimble to assist in temperature control, and the whole is surrounded by a layer of glass wool encased in a copper sheath so that only the mirror, which is jointed to the sheath by a band of epoxy-resin, remains exposed. The heat loss from the air in the jar into the thimble is balanced by an empirical adjustment of the voltage across an 0·2–3·0-W heater wound on the outside of the sheath. In this way the water bath, the sample under examination and the reference junctions of the thermopiles are maintained at the same temperature ($\pm$0·05°).

To determine the relative humidity of the air in the jar, the mirror is illuminated by a narrow beam of light projected from outside the water bath and is observed through a telescope; the fan is kept running because much sharper dew-points are obtained in moving air. The thimble is cooled until dew is observed and the compensating heater is set to balance the heat loss from the air into the thimble. The thimble is then slowly warmed and cooled at least five times while the temperature of each appearance and disappearance of dew is recorded. The mean of these temperatures is taken as the dew-point. Further sets of readings are taken at intervals of at least 40 min. until successive sets differ by less than 0·5% relative humidity and no systematic drift is evident.

Calibration and testing of the dew-point apparatus

The thermopiles were calibrated against precision thermometers, which had been checked against an N.P.L. standard thermometer. These thermopiles produce a potential of about 0·12 mV/deg. C difference and the potentiometer is accurate to closer than $\pm$0·01 mV so it is considered that the temperature measurements are accurate to within $\pm$0·1° which corresponds to about 0·5% R.H. at 100% R.H. and about 0·3% R.H. at 50% R.H. Dew-point depressions were calculated from tables of the vapour pressure of pure water.

The apparatus was tested by using saturated salt solutions and other solutions of known water activity as standards (Table I). In these tests the observed relative humidity of the air was always within 0·5% R.H. of that determined by Robinson & Stokes[5] and by Wexler & Hasegawa,[6] two very reliable sources. This precision is better than that often achieved by the use of solutions to control the relative humidity in enclosed atmospheres.[1]

Table I

The water activities of salt solutions as measured by the dew-point method

Salt	Concentration	Temperature					
		20°	25°	30°	35°	40°	50°
KCl	1·04M	0·968	0·964	0·967	0·964	0·963	—
KNO_3	Saturated	—	0·925	—	—	—	—
NaCl	4·82M	0·819	0·819	0·820	0·817	0·817	—
NaCl	Saturated	0·757	0·757	0·757	—	0·755	—
$Na_2Cr_2O_7,2H_2O$	,,	0·552	—	0·524	—	0·498	0·471
$MgCl_2,6H_2O$	,,	—	0·332	—	—	—	—

Determination of moisture content

Moisture content is not an easily defined characteristic of hygroscopic foods,[18] but by using standardised methods one can obtain reproducible results. The methods used in this investigation are used widely in commerce and in laboratories concerned with moisture in products.

In all the oven-drying methods, two or more replicate samples were dried in a mechanically ventilated oven in tared shallow aluminium containers, which were covered with tightly fitting lids and cooled in a desiccator before being weighed. For very wet samples a two-stage process, in which the material was partially dried at room temperature before being ground, was used.

Wheat and *sorghum* were ground in a Regent Maskin burr-type grinder and dried at 113° for 4 h.[18]

Groundnut kernels and *hazel nut kernels* were grated on a small food cutter and dried at 103° for 3 h. and then re-dried for successive periods of 1 h. until constant weight ($\pm$0·001%) was reached.

Groundnut meal was dried in the same way as the grated kernels.

Coffee beans were ground in a Regent Maskin burr-type grinder and dried at 105° for 4 h.

The moisture content of *pimento* and *black seed pepper* was determined by Mr. E. Brown of the Tropical Products Institute by the Dean & Stark distillation method.

Products examined

Groundnut kernels and *meal.*—A commercial sample of Nigerian kernels (series 1) and a sample of edible grade kernels purchased in England (series 2 and 3) were examined. In series 2, water activity determinations were made on whole kernels but in series 1 and 3, the kernels were grated so that they would reach equilibrium more quickly.

Wheat.—Two types of wheat were studied, No. 1 Manitoba, a ' strong ' Canadian wheat, and Cappelle, a typical English variety. The chemical and physical properties of these batches of wheat have been described in detail elsewhere.[19] The water activity measurements were made on whole grains.

Sorghum.—Samples of two varieties of sorghum, Bukura Mahemba and Wiru from Tanganyika were examined. Wiru is a white, very vitreous variety, and Bukura Mahemba a red, less vitreous variety. Water activity measurements were made on the whole grain.

Coffee.—A sample of undried robusta coffee beans was supplied directly from Uganda and

a further supply of dried Uganda robusta beans was obtained in England, as was a sample of Kenya arabica beans. Water activity was measured on whole beans and whole cherry.

Hazel nut kernels.—These measurements were made on a sample of kernels intended for confectionery. They were grated on a small food cutter before determination of water activity.

Black seed pepper.—Sub-samples from a single batch of Sarawak black seed pepper were first wetted and then dried to the required moisture content.

Pimento.—A 2·5 kg. sample of undried pimento was sent directly from Jamaica. Half of the sample was dried to about 8% moisture content and then used for determining the adsorption isotherm. The other half was dried in stages for the desorption isotherm. Water activity was determined on whole pimento as it was found that the volatile materials released on grinding condensed on the dew-point mirror and made it difficult to read the dew-point precisely.

Experimental procedure

For each product several 100–200-g. samples were adjusted to appropriate moisture contents either from a low initial moisture content by exposing them to very humid air[18] or from a high initial moisture by drying them in air at room temperature. These samples were then stored in sealed bottles at about 5° for several days to allow moisture equilibration. Moisture content was determined on sub-samples before and after measurement of water activity. When measurements were made at two or more temperatures the measurement at the first temperature was repeated at the end of the series to ensure that no drift in water activity had occurred.

Results and discussion

Table II*a–h* gives the water activity (a_w) and the moisture content expressed as a percentage of wet weight (% M.C.) of each sample examined. These results are summarised in Table III.

The water sorption isotherms of the two samples of groundnut kernels were virtually identical and, when the moisture content was calculated on an oil-free basis, were very similar to that of the extracted meal.

In both wheat and sorghum the moisture contents of the more vitreous types, Manitoba and Wiru, were lower, at the same water activity, than those of the less vitreous varieties, Cappelle and Bukura Mahemba. The difference was less than 1% moisture content but was about the same at both 25° and 35° and in the desorption and adsorption isotherms.

The two types of coffee beans had very similar water sorption isotherms but the coffee cherry contained slightly more water than the beans at the same water activity.

Only one sample each of hazel nut kernels, pimento and black pepper was examined.

Effect of temperature on water activity

One of the advantages of the dew-point method is that measurements of water activity can be made at different temperatures on the same sample. As the temperature is changed, the water content of the air, and therefore the moisture content of the sample, also changes, but for all the products studied the change of moisture content of a 100-g. sample with a change in temperature of 10° never exceeds the equivalent of 0·003 a_w.

Fig. 2 summarises the results on the effect of temperature on water activity in most of the products studied. There are marked differences between products but a temperature rise of 10° rarely causes an increase of more than 0·03 a_w.

In Fig. 3 a line summarising the present results on wheat is compared with lines derived from the results of Becker & Sallans[11] and of Gale[10] on wheat, and the prediction of Henderson[20] for a variety of products including wheat. The lines all indicate that the effect of temperature on water activity becomes very small as water activity approaches unity and that it is greatest at about the middle of the adsorption isotherm. The disagreement about the size of the effect may be attributable to differences in technique but further investigation would seem to be justified.

The safe moisture content for storage of many products falls as the temperature is raised through the normal storage range. This is probably due primarily to the fact that many fungi can grow at lower water activity at the higher temperatures. However the increase in water activity with temperature can also be large enough to be of practical importance. For example

Table II

Results of all the water activity and moisture content measurements

(% M.C. = % of wet wt. a_w = water activity)

(a) Groundnuts

Kernels

Series 1 Desorption		
% M.C.	a_w 20°	a_w 30°
13·0	0·893	0·895
10·6	0·837	0·835
9·6	0·811	0·810
9·1	0·795	0·794
7·5	0·724	0·727

Series 2 Desorption	
% M.C.	a_w 30°
37·4	0·992
31·5	0·983
25·4	0·972
19·8	0·949
15·7	0·915
11·6	0·860
9·8	0·815

Series 3 Desorption		
% M.C.	a_w 20°	a_w 30°
17·4	0·925	0·925
9·7	0·803	0·801
6·6	0·654	0·655
7·1	0·685	0·685
5·2	0·488	0·500
4·4	0·426	0·440
4·2	0·344	0·360

Series 1 Adsorption		
% M.C.	a_w 20°	a_w 30°
13·3	0·895	0·893
9·9	0·822	0·822
7·8	0·743	0·744
6·8	0·688	0·694
3·9	0·373	0·385

Extracted meal

Adsorption		
% M.C.	a_w 20°	a_w 30°
24·4	0·905	0·905
17·2	0·822	0·824
13·4	0·732	0·737
10·2	0·614	0·617

(b) Wheat

No. 1 Manitoba

Adsorption		
% M.C.	a_w 25°	a_w 35°
29·8	0·963	—
22·8	0·923	0·930
18·2	0·860	0·874
15·2	0·778	0·785
14·0	0·711	0·735
12·2	0·601	0·632
10·9	0·498	0·528

Desorption		
% M.C.	a_w 25°	a_w 35°
21·4	0·906	—
18·1	0·854	—
15·1	0·742	—
13·7	0·662	—
11·6	0·518	—
13·7	0·672	0·698
12·2	0·577	0·606
10·4	0·438	0·468

Cappelle

Adsorption		
% M.C.	a_w 25°	a_w 35°
23·3	0·930	—
18·5	0·854	0·859
15·4	0·748	0·760
14·3	0·691	0·715
13·0	0·602	0·633
11·5	0·487	0·518

Desorption		
% M.C.	a_w 25°	a_w 35°
22·1	0·912	—
17·8	0·827	—
14·9	0·702	—
12·7	0·549	—
11·1	0·435	—
14·2	0·652	0·676
13·5	0·605	0·635
11·9	0·478	0·511

(c) Sorghum

Bukura Mahemba

Adsorption		
% M.C.	a_w 25°	a_w 35°
7·9	0·222	0·247
10·7	0·487	0·513
13·6	0·687	0·710
16·6	0·853	0·862
21·5	0·937	0·939

Desorption		
% M.C.	a_w 25°	a_w 35°
22·9	0·950	0·951
18·6	0·877	0·884
15·5	0·765	0·775
13·5	0·631	0·654
11·0	0·452	0·486
9·3	0·324	0·354
6·6	0·179	0·200

Wiru

Adsorption		
% M.C.	a_w 25°	a_w 35°
7·9	0·266	0·293
10·0	0·456	0·485
12·2	0·628	0·656
15·3	0·807	0·824
19·7	0·923	0·925
21·8	0·956	0·958

Desorption		
% M.C.	a_w 25°	a_w 35°
23·8	0·970	0·696
20·4	0·926	0·926
17·3	0·861	0·871
14·6	0·720	0·742
12·3	0·588	0·615
9·8	0·414	0·445
7·5	0·255	0·282

(d) Coffee beans

Robusta, undried

Desorption		
% M.C.	a_w 25°	a_w 35°
17·2	0·826	0·829
14·6	0·776	0·778
12·3	0·696	0·703
10·1	0·585	0·597

Robusta, dried

Adsorption		
% M.C.	a_w 25°	a_w 35°
18·5	0·855	0·856
15·5	0·801	0·803
13·8	0·746	0·752
11·2	0·645	0·657
9·6	0·555	0·567

Desorption		
% M.C.	a_w 25°	a_w 35°
18·7	0·853	0·852
16·5	0·818	0·822
14·1	0·770	0·773
12·2	0·697	0·703
10·3	0·608	0·619

Arabica, dried

Adsorption		
% M.C.	a_w 25°	a_w 35°
24·4	0·905	0·905
21·5	0·884	0·884
16·7	0·821	0·822
13·2	0·729	0·737
10·6	0·609	0·619
8·1	0·423	0·446

Desorption		
% M.C.	a_w 25°	a_w 35°
21·6	0·887	0·887
18·3	0·857	0·859
13·9	0·773	0·774
11·4	0·682	0·693
8·9	0·526	0·540
7·9	0·453	0·472

(e) Coffee cherry

Desorption				
% M.C.	a_w 20°	a_w 30°	a_w 40°	a_w 50°
12·6	0·678	0·677	0·678	0·680
11·6	0·616	0·619	0·622	0·620
9·2	—	—	0·522	—
8·2	0·452	0·462	0·472	0·478

(f) Hazel nut kernels

Adsorption			
% M.C.	a_w 20°	a_w 30°	a_w 40°
7·3	0·842	0·842	0·840
5·7	0·773	0·775	0·770
5·2	0·737	0·737	0·735
5·0	0·710	0·713	0·719
4·5	0·692	0·692	0·688
3·5	0·575	0·590	0·584

(g) Black seed pepper

Desorption		
% M.C.	a_w 20°	a_w 30°
15·9	0·824	0·832
15·7	0·845	0·850
14·8	0·783	0·796
13·2	0·661	0·683
13·1	0·709	0·727
12·9	0·656	0·675
12·5	0·608	0·634
12·2	0·608	0·632
11·4	0·540	0·567
11·3	0·548	0·573

(h) Pimento

Desorption		
% M.C.	a_w 20°	a_w 30°
53·7	0·987	0·993
51·3	0·980	0·983
40·5	0·968	0·970
27·7	0·956	0·958
13·5	0·707	0·711

Adsorption		
% M.C.	a_w 20°	a_w 30°
24·1	0·943	0·943
18·8	0·882	0·884
16·0	0·844	0·849
13·9	0·743	0·758
9·7	0·480	0·509

Table III

Mean adsorption and desorption moisture contents at round values of water activity

Product	Temperature, °C	Water activity							
		0·40	0·50	0·60	0·70	0·80	0·90	0·95	0·98
Manitoba wheat	25	—	11·2	12·4	14·0	16·2	20·5	—	—
Cappelle wheat	25	—	11·8	13·2	14·6	16·9	21·0	—	—
Bukura Mahemba sorghum	25	10·1	11·4	12·7	14·2	16·0	19·2	—	—
Wiru sorghum	25	9·6	10·9	12·3	13·8	15·6	18·8	21·7	—
Robusta coffee beans	30	—	—	10·1	12·1	15·4	—	—	—
Arabica coffee beans	30	—	8·6	10·1	12·0	15·3	23·5	—	—
Robusta coffee cherry	30	—	9·0	11·1	13·1	—	—	—	—
Groundnut kernels	30	4·3	5·1	6·1	7·2	9·3	14·0	20	30
Groundnut extracted meal	30	—	—	—	12·3	16·3	23·5	—	—
Hazel nut kernels	30	—	—	3·6	4·7	6·3	—	—	—
Pimento	30	—	—	—	12·9	15·0	19·5	25·5	44
Black seed pepper	30	—	—	11·8	13·0	14·8	—	—	—

wheat at 0·7 a_w at 15° is safe for storage but, on the basis of the results in Fig. 2, the water activity of the same wheat at the same moisture content at 35° would be about 0·75. This latter combination of conditions allows some xerophilic fungi to grow quite rapidly.

Hysteresis

In many hygroscopic substances the water activity is higher when the sample reaches a given moisture content by sorption of water than when it does so by desorption. This hysteresis has been explained in a number of ways.[21, 22]

When products are exposed to air of constant relative humidity until they cease to change in weight, the observed hysteresis is often quite large, but this situation rarely occurs under practical conditions. Usually, especially when products are artificially dried, the outer layers of particles change in moisture content more rapidly than the inner layers, and during subsequent internal equilibration the direction of water exchange in the outer layers is reversed.[11] This appears to have occurred in some of the samples used in the present experiments and probably explains the low or even reversed hysteresis observed, for example, in coffee beans.

General formulae relating water activity to moisture content

The physical and chemical characteristics of stored food products are variable and complex and the free energy of sorbed water is reduced by several different mechanisms which vary in relative importance with the product, with the water activity and with the temperature. It

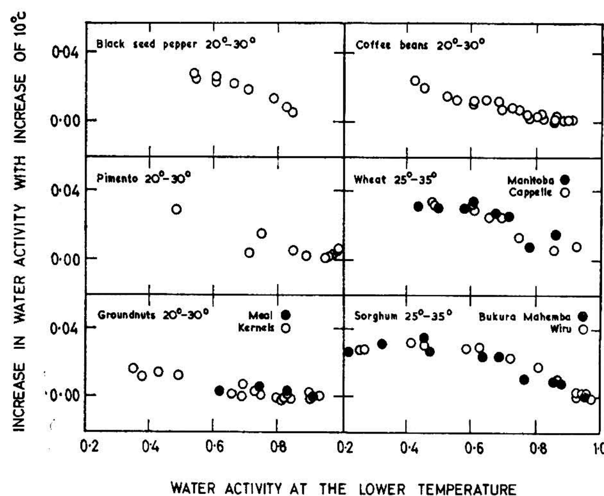

FIG. 2.—*Effect of temperature on water activity*

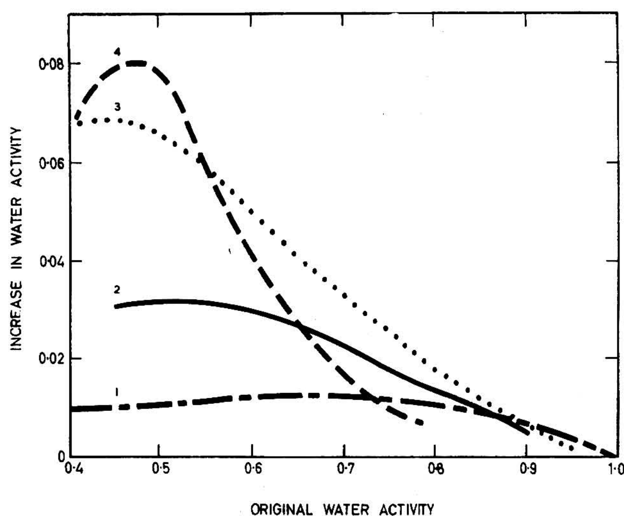

FIG. 3.—*Increase in water activity of wheat at constant moisture content with rise of* 10°

1. Henderson[20] 2. From Fig. 2
3. Becker & Sallans[11] 4. Gale[10]

would therefore be surprising if any relatively simple model were capable of explaining the water sorption behaviour of all products. However, a transformation which would enable water sorption isotherms to be expressed in a linear form would have considerable advantages in the interpretation of such data ; two transformations proposed for this purpose have been examined.

Smith[22] proposed the formula

$$w = a + b \ln (1 - a_w)$$

in which w is weight of water as a proportion of the total weight of the moist material and a and b are constants which are different for each material. He showed that it could be fitted to data for certain high polymers over the range $0·5a_w$ to $0·95a_w$. This formula was used by Becker & Sallans[11] who showed that it could be fitted to their data for wheat over the range $0·5a_w$ to $0·93a_w$.

Henderson[20] proposed the formula

$$1 - a_w = \exp (-kTM^n)$$

in which T is absolute temperature, M is moisture content as a percentage of the dry weight and k and n are constants, different for each material. He suggested that it held for a wide range of materials.

These two formulae were tested on the present results by plotting the functions of moisture content and water activity which should have given straight lines. Smith's equation fitted the results for wheat quite well but gave systematic deviations considerably greater than the estimated experimental error for some of the other products.

Henderson's equation resulted in systematic deviations from a straight line for all the results here and for results on cotton and Cellophane reported by other workers.[22] This equation also predicts that the effect of temperature on water activity at constant moisture content is the same in all products, with a maximum increase of about $0·012\ a_w/10°$ at about $0·6a_w$ (Fig. 3). The present observations (Fig. 2) do not substantiate this.

It is concluded that, although one or other of these formulae can be fitted to parts of the water sorption isotherms of some products, and Smith's equation is probably valid for the fairly pure high polymers for which it was designed, neither of them is universally applicable and there is considerable doubt as to the validity of the temperature term in Henderson's equation.

Acknowledgments

Many colleagues at this laboratory and elsewhere have had some hand in this investigation in the supply of materials and in the analyses. They include Mr. S. W. Pixton, Miss F. Ashwell

and Miss P. M. Davey who prepared some of the samples and determined some moisture contents; Mr. E. Brown of the Tropical Products Institute who determined the moisture contents of the pimento and black seed pepper samples; Mr. R. W. Disney who advised on the construction of the apparatus; and Miss H. M. Lee who assisted throughout the investigation.

Pest Infestation Laboratory
London Road
Slough, Bucks.

Received 22 June, 1964

References

[1] Scott, W. J., *Advanc. Food Res.*, 1957, **7**, 83
[2] Ingram, M., 'Microbial Ecology', *7th Symp. Soc. gen. Microbiol.* (London), 1957, p. 90 (Cambridge Univ. Press)
[3] Ludwig, D., *Physiol. Zoöl.*, 1945, **18**, 103
[4] Landrock, A. H., & Proctor, B. E., *Mod. Packag.*, 1951, **24**, 123, 130, 180
[5] Robinson, R. A., & Stokes, R. H., 'Electrolyte Solutions', 1955 (New York: Academic Press)
[6] Wexler, A., & Hasegawa, S., *J. Res. nat. Bur. Stand., Wash.*, 1954, **53**, 19
[7] Ives, N. C., *Agric. Engng St Joseph. Mich.*, 1952, **33**, 85
[8] Mossel, D. A. A., & Kuijk, H. J. L., *Fd Res.*, 1955, **20**, 415
[9] Hubbard, J. E., Earle, F. R., & Senti, F. R., *Cereal Chem.*, 1957, **34**, 422
[10] Gale, F. J., *J. Coun. sci. industr. Res. Aust.*, 1946, **19**, 187
[11] Becker, H. A., & Sallans, H. R., *Cereal Chem.*, 1956, **33**, 79
[12] Huckill, W. V., 'Drying of Grain', in Anderson, J. A., & Alcock, A. W. (ed.), 'Storage of Cereal Grains and Their Products', 1954, p. 410 (St. Paul, Minnesota: Amer. Ass. of Cereal Chemists)
[13] Lourenco, O. B., & Pucci, J. R., *Ann. Ass. quim. brasil.*, 1954, **13**, 15
[14] Fenton, F. C., *Agric. Engng St Joseph. Mich.*, 1941, **22**, 185
[15] White, R. T., *J. econ. Ent.*, 1957, **50**, 423
[16] Karon, M. L., & Hillery, B. E., *J. Amer. Oil Chem. Soc.*, 1949, **26**, 16
[17] Finn-Kelsey, P., & Hulbert, D. G., *Tech. Rep. Brit. elect. Res. Ass.*, W/T33, 1957, 26 pp. & suppl.
[18] Oxley, T. A., & Pixton, S. W., *J. Sci. Fd Agric.*, 1960, **11**, 315
[19] Pixton, S. W., Hyde, M. B., & Ayerst, G., *J. Sci. Fd Agric.*, 1964, **15**, 152
[20] Henderson, S. M., 1952, *Agric. Engng St Joseph. Mich.*, 1952, **33**, 29
[21] Brunauer, S., 'The adsorption of gases and vapours', Vol. 1, 'Physical Adsorption', 1943, p. 394 (Princeton Univ. Press)
[22] Smith, S. E., *J. Amer. chem. Soc.*, 1947, **69**, 646

ACID-FUME PEELING OF SOME FOOD PRODUCTS

By K. POPPER, F. S. NURY and W. L. STANLEY

It is possible to peel some fruit and vegetables by loosening the cellulosic connexion of the exocarp with acid fumes. An experimental procedure for a number of products is reported.

Introduction

Chemical peeling of fruits, vegetables and grains has been almost entirely limited to the the use of alkali,[1, 2] but an exception is the Mandarin orange segment from which short immersion in a 2·5% solution of hydrochloric acid removes remainders of albedo.[3] Peeling by what appears to be destructive dehydration with hot or cold concentrated sulphuric acid has also been suggested.[4, 5] While many products are at present peeled successfully by short contact

with dilute caustic soda solution (0·1–0·4%), others, such as carrots or potatoes, require lye solution of 10–15%[6] and still others including garlic and chestnuts cannot be peeled easily with lye at all.

Since the exocarp of many agricultural products consists predominantly of cellulosic tissues, acid hydrolysis should loosen the exocarp linkages and thus free the peel. Bases, on the contrary, cleave acetals with difficulty, if at all.

Experimental

A number of agricultural products (Table I) were exposed to fumes of concentrated hydrochloric acid. All experiments were conducted at ambient temperature and pressure in closed desiccators with 37% hydrochloric acid solution placed in a shallow container on the bottom: various products were held above the acid out of direct contact with the solution.

Table I

Treatment and time for acid fume peeling

Produce	Treatment after exposure			Time exposed to acid fumes							
	Wash or wet agitation	Brushing	Pressure or dry agitation	15 min.	45 min.	1 h.	4 h.	6 h.	12 h.	24 h.	48 h.
Apple	*	*		−	+	+	+	++			
Carrot		*		+	+						
Chestnut			*	−	−	−	−	−	+	+	
Garlic	*			−	−	−	+	+	+		
Ginger root		*				−	+				
Artichoke, Jerusalem		*									
Onion, Yellow Globe	*					−	+		+		
Onion, white, boiling (silver skin)	*					−	−	+	+		
Onion, red	*					−	−	+			
Pecan			*			−	−	−	−	+	+
Walnut, English			*				−	−		+	+
Wheat, kernel red	*	*	*			−	+	+	+		

− Does not peel + Peels readily ++ Edible portion attacked or partly destroyed

Samples were removed, washed and agitated periodically as required. Some products required soft brushing and/or gentle pressure (e.g., rubber finger) to remove the peel after exposure. Table I shows the time of exposure required and treatment needed for satisfactory peeling. Figs. 1 and 2 show some products peeled by the acid-fume method.

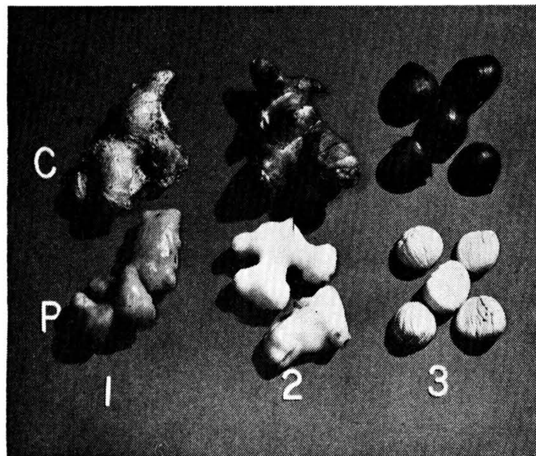

FIG. 1

1 ginger root 2 Jerusalem artichoke 3 chestnut

C control P peeled product

ห้องสมุด กรมวิทยาศาสตร์

Results

Peeling loss was very low in all cases, while with the nuts there was no loss. The anthocyanin pigments of red onion were accentuated. Peeled onion was no longer lachrymatory, but retained its typical onion flavour. Carrots turned more orange. Both Jerusalem artichoke and ginger root grew pink on prolonged exposure (6–8 h.).

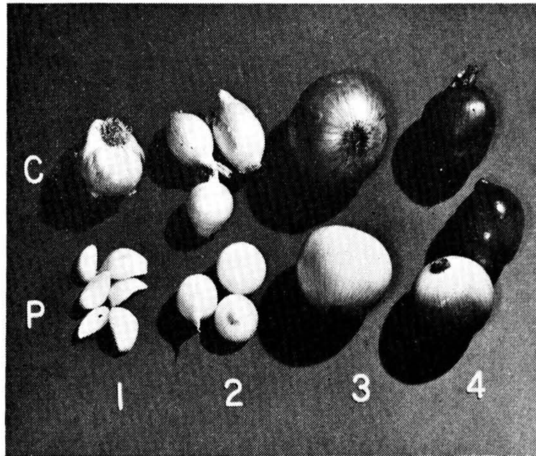

FIG. 2

1 garlic 2 boiling (silverskin) onion 3 Yellow Globe onion 4 red onion
C control P peeled product

Wheat debranned more easily when allowed to remain in boiling water for 5 min. after acid exposure. Peeled apples did not brown as fast as usual. No attempt was made to remove the seed coat from nuts. It was not possible to successfully peel potatoes or beans by this technique.

Western Regional Research Laboratory
Agricultural Research Service
U.S. Dept. of Agriculture
Albany 10, California

Received 4 June, 1964

References

[1] Bitting, K. G., 'Lye peeling', Nat. Canners Ass. Res. Lab., Bull. No. 10, 1917

[2] Dunlap, R. L., *Food Ind.*, 1944, **16**, 969, 1044

[3] 'Chemistry and Technology of Citrus, Citrus Products and Byproducts', 1962, Agric. Handbook No. 98, p. 32 (Washington, D.C.: U.S. Dept. Agric.)

[4] Ash, C. A., U.S.P. 1,453,781

[5] Mehring, E., Ger. P. 474,633

[6] Cruess, W. V., 'Commercial Fruits and Vegetable Products', 4th edn, 1958 (New York: McGraw-Hill Book Co.)

STUDIES ON THE NUTRITIONAL VALUE OF FOODS TREATED WITH γ-RADIATION. I.—Effects on some B-complex Vitamins in Egg and Wheat

By T. S. KENNEDY

By use of microbiological methods of assay, the effect of γ-radiation at doses of 0·5 Mrad and 5·0 Mrad on the content of some B-complex vitamins in frozen whole egg has been studied. For comparative purposes, analyses were also performed on egg treated by a heat-pasteurisation process designed to eliminate salmonellae food-poisoning organisms. No change was apparent in pantothenic acid, biotin and riboflavin after any of the treatments. A 24% loss of thiamine occurred at 0·5 Mrad and this was increased to 61% at 5·0 Mrads.

Similar analyses were made on Manitoba wheat irradiated at 0·02 Mrad, which is approximately the dose required for grain disinfestation, and also at ten times this dose. No apparent change was observed in the contents of nicotinic acid, thiamine, riboflavin, biotin and total vitamin B_6 after irradiation at 0·02 Mrad ; pantothenic acid showed a slight loss at this dose. At 0·2 Mrad a loss of 12% nicotinic acid, 11% pantothenic acid and 10% biotin occurred.

Introduction

Before the introduction of any radiation process for the treatment of food, its possible effect on wholesomeness with respect to both toxicity and nutritive value requires investigation.[1] Wheat and egg have been chosen for study of vitamin destruction since promising processes for the disinfestation of grain[2] and for the elimination of salmonellae from frozen egg[3] have been proposed. Furthermore, long-term animal feeding studies on these commodities have been undertaken at this laboratory in order to investigate their possible toxicity after irradiation ; such studies require that the test diets be nutritionally adequate. Some of the evidence obtained showing the non-toxicity of irradiated wheat has recently been published.[4]

Microbiological methods of vitamin assay were employed and the foods treated at the radiation doses recommended for practical application, and, in order to exaggerate any destruction and to conform to some of the diet treatments in the animal studies previously mentioned, also at doses ten times higher. In addition, the effect of a newly-proposed heat-pasteurisation process for eggs[5] was studied for comparative purposes. The vitamins involved were nicotinic acid, pantothenic acid, biotin, thiamine and riboflavin for both wheat and egg, plus vitamin B_6 in the case of wheat.

Experimental

Preparation and treatment of samples

Fresh whole egg was obtained from Messrs. J. Rannock Ltd., Bury St. Edmunds, Suffolk. After thorough homogenisation at the plant, part was heated at 149° F for 2·5 min. before freezing using the plate pasteuriser described by Heller *et al.*[5] The pasteurised egg was frozen to −15° C after being canned and was stored at this temperature until required. Other samples of the homogenised egg were irradiated with γ-radiation at 0·5 and 5·0 Mrads in the Spent Fuel Rod Assembly at A.E.R.E., Harwell ; the dose-rate was approximately 1 Mrad/h. The egg was maintained at −15° C during transport, irradiation and storage. The egg used as control was untreated except with respect to the freezing procedure. Vitamin analyses were performed directly in the samples of thawed egg.

Manitoba No. 2 wheat was thoroughly mixed in a butter churn and the moisture content determined as 11%. Part of the wheat was irradiated at 20°C in a 10,000-curie cobalt-60 source at 0·02 Mrad and 0·2 Mrad at a dose-rate of approximately 0·03 Mrad/h. The untreated and irradiated wheat was held in store for 3 months at room temperature and then milled. The flour was mixed in a butter churn and samples were defatted by Soxhlet extraction, dried and thoroughly mixed and stored in airtight tins at 2°C until required. Moisture content of the samples was estimated before analyses to allow results to be expressed on a moisture-free (and also fat-free) basis.

Microbiological assay methods

Samples were assayed in a multiple assay thus giving a direct comparison between treatments. From each sample extracts were prepared in triplicate and each extract assayed at three levels.

For egg and wheat assays for nicotinic acid, pantothenic acid and biotin the organism *Lactobacillus plantarum* NCIB 6376 was used and for riboflavin assays on wheat, *Lactobacillus casei* NCIB 8010. Unsatisfactory results were obtained with *Lb. casei* for riboflavin in egg and the more sensitive method of Kornberg *et al.*[6] with *Streptococcus faecalis* var. *liquefaciens* NCIB 7432 was used. Stock cultures of lactobacilli were maintained by monthly subculture in tomato juice agar stabs and stored at 2° C until required. The general assay methods using lactobacilli were essentially the same as those described by Barton-Wright.[7] Acid hydrolysis of extracts was carried out in an autoclave at 15 lb. p.s.i. with 70 ml. of N-sulphuric acid for 1 h. for nicotinic acid, 50 ml. of 3N-sulphuric acid for 30 min. for biotin, and 35 ml. of 0·1N-hydrochloric acid for 15 min. for riboflavin. With these vitamins interfering substances were removed by the Strong & Carpenter technique.[8] Pantothenic acid extracts were hydrolysed by an enzymic method.[9] The basal media used for nicotinic acid and riboflavin in wheat were those recommended by the Society of Public Analysts,[10] but the riboflavin-free yeast supplement was prepared using Florisil adsorbent,[11] while for biotin the medium was that of Wright & Skeggs,[12] and for pantothenic acid that of Barton-Wright.[13]

Thiamine was assayed by the method of Hoff-Jorgensen & Hansen[14] and total vitamin B_6 by the method of Atkin *et al.*[15]

Growth was measured turbidimetrically in a Hilger model H.810 absorptiometer with a 610 mμ filter.

Statistical analysis

Results were submitted to an analysis of variance using the method described by Finney[16] for parallel line assays. Mean squares of deviations from linearity, parallelism, preparation differences and slope were tested against error with a variance ratio test for significance. Potency estimates were computed and a direct comparison of the treatments was made.

Results

The results for frozen whole egg are shown in Table I and for wheat in Table II. Vitamin losses which are significant ($P = 0·05$) are given in Table III, expressed as percentage of control—these are the combined losses obtained by comparison of the treatments with the control and not from the combined potency figures, thus allowing differences in the potency of the untreated samples to be neglected.[16]

Discussion

A microbiological method of vitamin assay is based on the use of a particular micro-organism under conditions where the vitamin is the limiting factor for growth. It is conceivable that irradiation of a vitamin could give breakdown products which are biologically active and therefore interfere with the assay procedure. This problem has been previously investigated[17] with respect to a number of water-soluble vitamins and it was concluded that microbiological methods of assay were to be recommended in preference to physico-chemical procedures, and during the course of this present work no evidence of interference was apparent.

For thiamine, which was expected to suffer some radiation destruction, *Kloeckera brevis* was chosen as test organism since it will not utilise either the thiazole or the pyrimidine moieties of thiamine[14] which might well be formed in the degradation products after irradiation.[18]

In spite of the fact that freezing has been shown to exert a protective effect on the radiation destruction of thiamine,[19] significant losses occurred in frozen whole egg, thus confirming the comparatively high radiation sensitivity of this vitamin as has been observed with other foods.[18, 20] However, a 24% loss recorded at the practical dose of 0·5 Mrad required for a radiation process aimed at elimination of salmonellae should not detract from its usefulness. Thiamine loss is particularly high after a radiation-sterilisation dose which is generally accepted

Table I

Effect of heat or radiation treatment on some B-complex vitamins in frozen whole egg

(figures in parentheses indicate 95% confidence limits)

Vitamin	Sample no.	Untreated (control)	Heat pasteurised (149° F for 2·5 min.)		Irradiated 0·5 Mrad		Irradiated 5·0 Mrads	
		Potency, µg./g.	Potency, µg./g.	% of control	Potency, µg./g.	% of control	Potency, µg./g.	% of control
Nicotinic acid	1	0·63	0·56	89	0·54	85	0·56	88
		(0·50–0·80)	(0·45–0·71)	(75–105)	(0·43–0·68)	(72–101)	(0·44–0·70)	(74–103)
	2	0·62	0·65	104	0·60	96	0·49	79
		(0·53–0·73)	(0·56–0·76)	(94–117)	(0·51–0·69)	(86–107)	(0·42–0·57)	(71–88)
Pantothenic acid	3	17·4	18·2	104	17·7	101	17·0	97
		(16·3–18·6)	(17·0–19·4)	(99–110)	(16·5–18·9)	(97–106)	(15·8–18·2)	(92–103)
	4	17·3	17·5	101	17·8	103	17·7	102
		(16·6–18·0)	(16·8–18·2)	(98–104)	(17·1–18·6)	(100–106)	(17·0–18·4)	(99–105)
Thiamine	5	1·53	1·48	97	1·20	79	0·58	38
		(1·35–1·73)	(1·31–1·68)	(89–106)	(1·06–1·36)	(72–86)	(0·53–0·63)	(35–41)
	6	1·53	1·45	95	1·13	72	0·60	39
		(1·40–1·67)	(1·32–1·58)	(89–101)	(1·03–1·23)	(68–77)	(0·51–0·71)	(34–45)
Biotin	7	0·19	0·19	101	0·19	101	0·21	113
		(0·16–0·22)	(0·16–0·22)	(90–114)	(0·16–0·22)	(90–114)	(0·18–0·25)	(100–126)
	8	0·20	0·20	100	0·20	99	0·21	104
		(0·18–0·21)	(0·18–0·21)	(95–106)	(0·18–0·21)	(94–104)	(0·19–0·22)	(99–109)
Riboflavin	9	3·04	3·12	103	3·05	100	2·98	98
		(2·87–3·22)	(2·95–3·31)	(100–105)	(2·87–3·23)	(97–103)	(2·81–3·12)	(95–101)
	10	2·98	3·04	102	3·04	102	2·91	98
		(2·83–3·13)	(2·89–3·19)	(99–105)	(2·89–3·19)	(100–104)	(2·77–3·06)	(96–100)

Table II

Effect of γ-radiation on some B-complex vitamins in Manitoba wheat stored at room temperature after treatment for 3 months

(figures in parentheses indicate 95% confidence limits)

Vitamin	Sample no.	Untreated (control)	Irradiated 0·02 Mrad		Irradiated 0·2 Mrad	
		Potency, µg./g.*	Potency, µg./g.*	% of control	Potency, µg./g.*	% of control
Nicotinic acid	1	53·8	55·0	102	45·9	85
		(51·8–56·0)	(52·9–57·2)	(99–105)	(44·2–47·8)	(83–88)
	2	50·0	48·4	97	45·9	91
		(46·4–53·2)	(45·2–52·8)	(93–102)	(42·5–48·6)	(87–96)
Pantothenic acid	3	9·16	9·15	100	7·86	86
		(8·37–10·07)	(8·31–10·05)	(93–107)	(7·13–8·65)	(80–92)
	4	10·38	9·76	94	9·45	91
		(9·76–11·05)	(9·17–10·39)	(90–98)	(8·88–10·06)	(87–95)
Thiamine	5	4·80	4·80	100	4·93	103
		(4·23–5·42)	(4·24–5·43)	(92–109)	(4·35–5·57)	(94–112)
	6	4·70	4·78	102	4·82	103
		(4·38–5·03)	(4·46–5·07)	(97–107)	(4·50–5·16)	(98–108)
Riboflavin	7	0·62	0·59	95	0·66	105
		(0·57–0·69)	(0·54–0·65)	(89–101)	(0·59–0·72)	(98–112)
	8	0·59	0·61	94	0·60	101
		(0·55–0·64)	(0·57–0·66)	(89–110)	(0·55–0·64)	(96–107)
Biotin	9	0·12	0·12	99	0·11	90
		(0·11–0·14)	(0·11–0·14)	(91–108)	(0·10–0·12)	(82–98)
Total vitamin B_6	10	4·59	4·48	98	4·45	97
		(4·30–4·89)	(4·20–4·78)	(93–102)	(4·18–4·75)	(93–101)

* Fat- and moisture-free

Table III

Significant vitamin losses ($P = 0{\cdot}05$) expressed as % of control (untreated) and calculated from the combined results of assays performed on frozen whole egg and wheat after various treatments

(95% confidence limits in parentheses)

Vitamin	Frozen whole egg			Wheat	
	Heat-pasteurised 149° F for 2·5 min.	Irradiated 0·5 Mrad	Irradiated 5·0 Mrads	Irradiated 0·02 Mrad	Irradiated 0·2 Mrad
Nicotinic acid	*	*	18 (11–25)	*	12 (8–15)
Pathothenic acid	*	*	*	4 (1–8)	11 (7–14)
Thiamine	*	24 (18–29)	61 (57–66)	*	*
Riboflavin	*	*	*	*	*
Biotin	*	*	*	*	10 (2–17)
Total vitamin B_6				*	*

* No loss

to lie between 4 and 5 Mrads for most foods. The degradation products of this vitamin have proved difficult to identify,[20] but if any are shown to be characteristic of radiation destruction, then this might provide a basis for a method of detecting whether food has been irradiated or not for the purpose of inspection and control of a commercial process. The extent of its destruction with increasing dose has already been correlated with certain organoleptic changes in irradiated meat.[19]

With wheat, a radiation dose of approximately 0·02 Mrad, as recommended for disinfestation,[2] would not be expected to cause any destruction of the vitamins studied, including thiamine. The absence of destruction is particularly important if such a process is to be applied on a large scale, particularly in countries where cereals are the staple diet. The stability of thiamine in wheat confirms some previous work,[21] although losses have been reported in irradiated wheat flour.[22]

Acknowledgments

The author gratefully acknowledges the interest of Mr. F. J. Ley in the course of this work and thanks Mrs. M. J. Jones for excellent technical assistance and Mr. T. F. J. Hobson for the statistical analysis.

Isotope Research Division
Wantage Research Laboratory (A.E.R.E.)
Wantage, Berks.

Received 22 June, 1964

References

1 Ley, F. J., & Hickman, J. R., *Research, Lond.*, 1960, **13**, 193
2 Cornwell, P. B., & Bull, J. O., *J. Sci. Fd Agric.*, 1960, **11**, 754
3 Ley, F. J., Freeman, B. M., & Hobbs, B. C., *J. Hyg., Camb.*, 1963, **61**, 515
4 Hickman, J. R., McLean, D. L. A., & Ley, F. J., *Food & Cosmetics Toxicol.*, 1964, **2**, 15
5 Heller, C. L., Roberts, B. C., Amos, A. J., Smith, H. E., & Hobbs, B. C., *J. Hyg., Camb.*, 1962, **60**, 135
6 Kornberg, H. A., Langdon, R. S., & Cheldelin, V. H., *Analyt. Chem.*, 1948, **20**, 81
7 Barton-Wright, E. C., 'The Microbiological Assay of the Vitamin B-Complex and Amino Acids', 1952 (London: Sir Isaac Pitman & Sons Ltd.)
8 Strong, F. M., & Carpenter, L. E., *Industr. Engng Chem. (Anal.)*, 1942, **14**, 909
9 Novelli, G. D., & Schmetz, F. J., *J. biol. Chem.*, 1951, **192**, 181
10 'Report on the Microbiological Assay of Riboflavin and Nicotinic Acid', *Analyst*, 1946, **71**, 397
11 Ass. of Vitamin Chemists, Inc., 'Methods of Vitamin Assay', 1951, 2nd edn (New York Interscience Publishers, Inc.)
12 Wright, L. D., & Skeggs, H. R., *Proc. Soc. exp. Biol., N.Y.*, 1944, **56**, 95
13 Barton-Wright, E. C., *Analyst*, 1945, **70**, 283
14 Hoff-Jorgensen, E., & Hansen, B., *Acta chem. scand.*, 1955, **9**, 562
15 Atkin, L., Chultz, A. S., Williams, W. L., & Frey, C. N., *Industr. Engng Chem. (Anal.)*, 1943, **15**, 141
16 Finney, D. J., 'Statistical Method in Biological Assay', 1952 (London: Charles Griffin & Co. Ltd.)
17 Sjostedt, M., & Ericson, L-E., *Acta chem. scand.*, 1962, **16**, 1989
18 Ziporin, Z. Z., Kraybill, H. F., & Thach, H. J., *J. Nutr.*, 1957, **63**, 201
19 Wilson, G. M., *J. Sci. Fd Agric.*, 1959, **10**, 295
20 Groninger, H. S., & Tappel, A. L., *Food Res.*, 1957, **22**, 519
21 Vincent, O., *J. of Hyg., Epidemiol., Microbiol. Immunol., Prague*, 1961, **5**, 248
22 Egiazorov, G. M., *Voprosy Pitaniya*, 1960, **19**, 54

INSECTICIDAL ACTIVITY OF PYRETHRUM EXTRACT AND ITS FOUR INSECTICIDAL CONSTITUENTS AGAINST HOUSE FLIES. VI.*—Relative Toxicity of Pyrethrin I and Pyrethrin II against Four Strains of House Flies

By R. M. SAWICKI and M. ELLIOTT

Freshly reconstituted samples of pyrethrins I and II were tested against four strains of house flies. Pyrethrin II was 1·21–1·50 times more toxic than pyrethrin I, 24 h. after treatment, and 1·09–1·54 times more toxic, 48 h. after treatment. The strain of flies and the method of immobilising the insects before treatment (CO_2 or chilling) had little effect on the relative toxicity of the two esters. Two strains resistant to organophosphorus insecticides were very strongly resistant to knock-down but not to kill by the pyrethrins. The two esters were stable for 3 months when stored as 5% w/v solutions in darkness at −20° ; only pyrethrin II lost some of its insecticidal activity (23%) when left in acetone solution in daylight for 5 days at 15–20°.

Introduction

The aim of this work was to discover why American workers[1–3] found pyrethrin I to be from 1·7 to 4·3 times more toxic to house flies (*Musca domestica* L.) than pyrethrin II, whereas Sawicki and colleagues at Rothamsted[4, 5] always found pyrethrin II 1·3–1·6 times more toxic than pyrethrin I.

Differences in bioassay technique (Table I), such as spraying in odourless kerosene or topical application of drops, might produce such differences, but Chang & Kearns[3] and Sawicki[4, 5] both used topical application of acetone solutions. Therefore using this one method, the following factors which might affect the relative toxicities of pyrethrin I and II were investigated: (*a*) the strain of flies used, (*b*) the method of immobilisation before treatment (anaesthesia with carbon dioxide or chilling), and (*c*) the storage conditions for the two esters.

Table I

Toxicity of pyrethrin II relative to pyrethrin I against M. domestica *L.*

Authors	Method of preparation of constituents	Strain of house fly	Method of treatment and solvent used	Relative toxicity of pyrethrin II (pyrethrin I = 100)
Gersdorff[1]	Reconstitution	Not stated	Turntable method	23
Incho & Greenberg[2]	Reconstitution	Not stated	Odourless kerosene	39
Sawicki *et al.*[4]	Reconstitution and chromatography	Rothamsted normal	Topical application acetone	130–150
Sawicki[5]	Reconstitution and chromatography	Rothamsted normal	Topical application acetone	161
Chang & Kearns[3]	Chromatography	Wilson	Topical application	58
Matsui & Meguro[15]	Reconstitution	Not stated	Turntable method	31

Experimental

Materials

Pyrethrin I and pyrethrin II were reconstituted especially for these tests from pyrethrolone (purified as its hydrate) and naturally derived chrysanthemic and pyrethric acids, as described earlier.[4, 5, 6] Their structure and purity were again confirmed by chemical and physical methods already described.[7, 8] In addition, evidence for authenticity was obtained from their nuclear magnetic resonance spectra.[9] No absorption due to the isopyrethrolone side-chain[10] ($CH:CH·CH:CH·CH_3$), produced by thermal isomerisation, was detected.

The esters were dissolved in acetone to give stock solutions of 5% w/v 1 h. after preparation and then stored in the dark at −20° as before.[4] Throughout the tests the stock solutions were at room temperature only while dilutions were prepared. The flies of the various strains were treated with the same serial dilutions. In one experiment (17.2.64) to test the stability of

* Part V: *J. Sci. Fd Agric.*, 1962, **13**, 591

pyrethrin I and pyrethrin II in daylight at room temperature (15–20°), each ester (4 ml. of a 0·25% solution) was left in a glass-stoppered test tube in the laboratory on the bench, away from direct daylight for 5 days, during which time the sky was continuously cloudy.

The strains of flies used in the tests were: (*a*) the Rothamsted normal strain. Earlier[4] this strain had been found susceptible to all commonly used insecticides, but at the time of these tests it was moderately resistant to dieldrin and DDT, but not to pyrethrum extract or to the organophosphorus insecticides. Presumably the colony was contaminated in the previous year by stray resistant flies in the insectary; (*b*) the Regular strain from Gainsville, Florida, U.S.A., slightly resistant to DDT, but not to pyrethrins; (*c*) the Cradson P strain (also from Gainsville) selected in the U.S.A. for resistance to parathion but unselected since receipt at Rothamsted; and (*d*) the SKA strain, a cross between two diazinon-resistant strains, Sacca A (Italian) and 203a (Danish), very resistant to diazinon and the chlorinated insecticides.[11]

Methods

The bioassay technique has already been described.[12] Mortality was recorded at 24 and 48 h. after treatment. In most experiments the flies were lightly chilled in dishes in a refrigerator for 2–3 min. immediately before treatment. In one experiment (12.12.63) the flies were chilled for 5 min. or longer, until their legs were completely withdrawn under their thoraces (a sign of considerable chilling), and on 18.12.63 some of the flies were lightly anaesthetised with carbon dioxide before treatment.

The toxicity tests followed the general design of the probit assay.[13] Each of the four strains was treated on three occasions with the two pyrethrins. Each ester was tested at seven or more concentrations, three replicates of 15 flies per concentration; the dilution factor was 1·33. The toxicity of pyrethrin II relative to pyrethrin I at LD_{50} in each test, and the weighted mean toxicity of pyrethrin II relative to pyrethrin I for each strain, were tested for significance. The weighted mean relative toxicity of pyrethrin II for the Rothamsted strain was from eight experiments, done in this series of tests on this strain. Except where specified, χ^2 was not significant at $P = 0{\cdot}05$. The relative toxicity of pyrethrin II was significantly different when t was greater than 1·96 at $P = 0{\cdot}05$. The log dose–probit lines are referred to as ld–p lines.

The LD_{50} values are given throughout in μg. of insecticide per fly; the average weight of a female fly was approx. 20 mg.

Results

(i) *ld–p lines of the four strains*

Within each strain, the LD_{50} figures from the different experiments were reasonably constant (Table II*a* and *b*); they varied most with the Rothamsted normal strain, where the highest value was 1·90 times greater than the lowest. The differences between the LD_{50} figures from each test at 24 and 48 h. were small and not consistent in all except the Rothamsted normal strain, for which LD_{50} figures at 48 h. for pyrethrin I were smaller than the corresponding figures for 24 h., i.e. in that strain, the end-point occurred later for pyrethrin I than pyrethrin II. The ld–p lines of pyrethrin II with the Rothamsted normal strain were slightly steeper than those for pyrethrin I, but this difference was smaller than in previous tests.[4] With the other strains, the differences between the slopes of the ld–p lines for pyrethrin I and II were either small or not consistent. In one experiment (SKA, 17.11.63) the slope for pyrethrin I was unusually steep (Table II*a* and *b*).

The four strains differed somewhat in their susceptibility and their knock-down response to the pyrethrins. The decreasing order of susceptibility 24 h. after treatment was: Rothamsted normal (average LD_{50} pyrethrin I 0·58 μg./fly; pyrethrin II 0·47 μg./fly), Regular (average LD_{50} pyrethrin I 0·84 μg./fly; pyrethrin II 0·66 μg./fly), Cradson P (average LD_{50} pyrethrin I 1·08 μg./fly; pyrethrin II 0·77 μg./fly) and SKA (average LD_{50} pyrethrin I 1·40 μg./fly; pyrethrin II 0·93 μg./fly). The Rothamsted normal strain was on average 1·45, 1·86 and 2·41 times more susceptible to pyrethrin I, and 1·40, 1·64 and 1·98 times more susceptible to pyrethrin II than the Regular, Cradson P and the SKA strain. The main difference between

Table II

Results of bioassays of pyrethrin I and pyrethrin II against four strains of house flies

Strain	Date of experiment	Pyrethrin I b ± S.E.		Pyrethrin I Log (LD_{50} × 10³) ± S.E. μg./fly		Pyrethrin II b ± S.E.		Pyrethrin II Log (LD_{50} × 10³) ± S.E. μg./fly		Relative toxicity of py. II (py. I = 100)	test[a]	Weighted mean relative toxicity of py. II (py. I = 100)
(a) 24-hour results												
Rothamsted normal	17.11.63	4·45	0·47	2·596	0·025	4·57	0·54	2·517	0·025	120	+	
	2.12.63	3·40	0·42	2·804	0·031	4·28	0·48	2·692	0·024	129	+	121 ± 4[b]
	5.12.63	3·16	0·34	2·748	0·029	3·67	0·40	2·648	0·025	126	+	
	10.12.63	4·11	0·40	2·781	0·024	4·49	0·45	2·733	0·024	112	−	
Regular	2.12.63	4·82	0·58	2·963	0·023	7·28	0·98	2·822	0·019	138	+	123 ± 6
	5.12.63	3·95	0·41	2·845	0·026	5·25	0·49	2·816	0·021	107	−	
SKA	17.11.63	10·06	1·55	3·034	0·016	5·53	0·65	2·947	0·022	122	+	
	2.12.63	3·94	0·53	3·270	0·030	3·34	0·66	3·045*	0·048	189	+	130 ± 7
	10.12.63	6·65	2·47	3·102**	0·056	7·27	1·02	2·911	0·019	164	+	
Cradson P	17.11.63	3·36	0·37	2·958	0·031	2·13	0·18	2·801	0·037	144	+	
	5.12.63	3·12	0·31	3·060	0·029	2·82	0·29	2·838	0·030	167	+	150 ± 8
	10.12.63	3·12	0·31	3·064	0·030	3·00	0·29	2·990	0·029	118	−	
(b) 48-hour results												
Rothamsted normal	17.11.63	3·24	0·35	2·585	0·032	3·81	0·46	2·525	0·029	115	−	
	2.12.63	3·94	0·53	2·762	0·032	3·34	0·66	2·718	0·025	108	−	
	5.12.63	2·34	0·30	2·572	0·040	3·10	0·38	2·715	0·029	72[c]	−	109 ± 4[b]
	10.12.63	3·60	0·36	2·751	0·026	4·58	1·07	2·738***	0·047	103	−	
Regular	17.11.63	2·19	0·31	2·732	0·043	2·97	0·28	2·691	0·031	110	−	
	2.12.63	3·51	0·67	2·932*	0·049	4·68	0·51	2·864	0·023	117	−	111 ± 7
	5.12.63	3·49	0·38	2·754	0·030	3·34	0·32	2·774	0·026	96	−	
SKA	17.11.63	8·82	1·40	3·094	0·018	4·42	0·48	2·525	0·029	141	+	
	2.12.63	2·80	0·37	3·257	0·041	4·13	0·40	2·980	0·024	189	+	
Cradson P	17.11.63	2·78	0·26	3·045	0·033	1·97	0·17	2·796	0·040	177	+	
	10.12.63	2·91	0·29	3·053	0·031	3·82	0·38	2·933	0·026	132	+	147 ± 10

* χ^2 not significant at P = 0·02 ** χ^2 significant = 14·58 *** χ^2 significant = 16·99
[a] + py. II significantly more toxic than py. I − py. II not significantly more toxic than py. I
[b] mean of eight tests [c] py. I significantly more toxic than py. II

the strains was their response to the knock-down effect of the two pyrethrins. The flies of the Rothamsted normal and the Regular strains were rapidly knocked-down by the two pyrethrins, but the flies of the two strains resistant to organophosphorus insecticides (Cradson P and SKA) were very resistant to knock-down and showed the first signs of paralysis only 1 h. or more after treatment, even when treated with 10 μg./fly.

(ii) *Relative toxicity of pyrethrin II and pyrethrin I*

Tables II*a* and *b* show the toxicity of pyrethrin II relative to pyrethrin I in each test and its weighted mean relative toxicity for each strain of flies. By definition the relative toxicity of pyrethrin I was 100 in all the tests.

Pyrethrin II was significantly more toxic than pyrethrin I 24 h. after treatment against the four strains of flies. The weighted mean relative toxicity of pyrethrin II was: Rothamsted normal, 121 ± 4; Regular, 123 ± 6; SKA, 130 ± 7 and Cradson P, 150 ± 8. The relative toxicity of pyrethrin II at 48 h. was less uniform; pyrethrin II was significantly more toxic against the Cradson P, the SKA and the Rothamsted normal strains (weighted mean relative toxicities: 147 ± 10, 154 ± 9 and 109 ± 4, respectively), but did not differ significantly from pyrethrin I against the Regular strain (weighted mean relative toxicity, 111 ± 7).

(iii) *Effect of methods of immobilising the flies before treatment*

Table III shows that the method of immobilising the flies before treatment had no effect on the relative toxicity of the pyrethrins, and that slight chilling or CO_2 anaesthesia gave no significant difference at LD_{50}. Considerable chilling may have increased the susceptibility of the flies to the pyrethrins, at least during the first 24 h. Lightly chilled flies needed 23% more pyrethrin I and 24% more pyrethrin II than heavily chilled flies for a 50% kill.

(iv) *Stability of pyrethrins I and II to light at room temperature*

Samples left in daylight in the laboratory for 5 days were tested against stock solutions kept in the dark at −20° on flies of the Rothamsted normal strain. Table IV shows that

Table III

Results of bioassays of pyrethrin I and pyrethrin II against house flies (Rothamsted normal) immobilised by chilling or CO_2

Method of immobilising	Time after treatment, h.	Pyrethrin I $b \pm$ S.E.		Pyrethrin I Log ($LD_{50} \times 10^3$) $\pm$S.E. μg./fly		Pyrethrin II $b \pm$ S.E.		Pyrethrin II Log ($LD_{50} \times 10^3$) $\pm$S.E. μg./fly		Relative toxicity of py. II (py. I=100)	*t* test[a]
Normal chilling	24	2·91	0·43	2·868	0·039	4·20	0·49	2·716	0·025	142	+
,, ,,	48	3·33	0·42	2·730	0·030	4·22	0·54	2·583	0·028	140	+
Heavy chilling	24	4·13	0·56	2·776	0·028	5·93	0·82	2·628	0·024	141	+
,, ,,	48	2·99	0·47	2·639	0·037	4·37	0·64	2·563	0·031	119	—
Normal chilling	24	3·43	0·32	2·741	0·027	4·79	0·48	2·751	0·020	98	—
CO_2 anaesthesia	24	3·24	0·49	2·767*	0·044	4·80	0·49	2·695	0·023	118	—

* χ^2 not significant at P = 0·02

[a] + py. II significantly more toxic than py. I — py. II not significantly more than py. I

Table IV

24 h. results of a bioassay of pyrethrin I and pyrethrin II, exposed to light for 5 days at 15–20°, and stored in the dark at −20°, against house flies of the Rothamsted normal strain

Storage conditions	Pyrethrin I $b \pm$ S.E.		Pyrethrin I log ($LD_{50} \times 10^3$) $\pm$S.E. μg./fly		Pyrethrin II $b \pm$ S.E.		Pyrethrin II log ($LD_{50} \times 10^3$) $\pm$S.E. μg./fly		Relative toxicity of py. II (py. I=100)	*t* test[a]
Exposed to light at 15–20°	3·45	0·59	2·650	0·027	3·57	0·44	2·524	0·028	134	+
In dark at −20°	3·06	0·35	2·653	0·032	3·61	0·40	2·414	0·027	174	+

[a] + py. II significantly more toxic than py. I

pyrethrin I lost no insecticidal activity when left exposed to light, whereas pyrethrin II lost about a quarter (23%) of its toxicity; this loss was statistically significant.

The two samples of pyrethrin II (exposed to light and stored in darkness) were significantly more toxic than the corresponding samples of pyrethrin I, and the relative toxicity of pyrethrin II stored in dark to pyrethrin I (174), was greater than the relative toxicity of pyrethrin II kept in daylight (134). For unknown reasons, the relative and absolute toxicity of pyrethrin II stored in the dark was much more than in the other tests of this series.

Discussion

This work confirms earlier results showing pyrethrin II to be 1·3–1·6 times more toxic than pyrethrin I.[4, 5]

The relative toxicities of pyrethrin I and pyrethrin II against the four strains of flies differed little; pyrethrin II was between 1·21 and 1·50 times more toxic than pyrethrin I against the four strains, 24 h. after treatment; 48 h. after treatment it was significantly more toxic (between 1·09 and 1·54 times) than pyrethrin I against three of the strains, and at least as toxic as pyrethrin I against the fourth strain (1·11 times). Pyrethrin I was significantly more toxic than pyrethrin II in only one of 31 results. The differences in the relative toxicities of the two pyrethrins against the four strains were surprisingly small, considering the large variations in their response to other insecticides; the relative toxicity of pyrethrin II was slightly greater with the two strains resistant to organophosphorus insecticides (SKA and Cradson P) than with the other two strains (Rothamsted normal and Regular), especially 48 h. after treatment. At LD_{50} the resistant strains were between 0·75–0·50 times as susceptible to the pyrethrins as the Rothamsted normal strain; they were also very resistant to knock-down and in this differed very considerably from the other two strains.[14] Resistance to knock-down, but not to killing property was associated in these strains with resistance to organophosphorus and chlorinated insecticides.

The method of immobilising the flies during treatment had no effect on the relative toxicity of the pyrethrins, and the ld–p lines. Strong chilling possibly increased the susceptibility of the flies to the pyrethrins, at least during the first 24 h. after treatment, but did not alter the relative toxicities.

The method of storage of the pyrethrins was the only factor to affect appreciably the relative and absolute toxicity of pyrethrin II. Under the conditions used (in darkness at $-20°$) the pyrethrins lost no insecticidal activity over 3 months, because the LD_{50} values for the two compounds, 3 days after being prepared, did not differ appreciably from the values 3 months later. However, after 5 days in daylight at 15–20° the LD_{50} of pyrethrin II fell by 23%, but that of pyrethrin I did not change, and this resulted in a decrease in the relative toxicity of pyrethrin II from 174 to 134.

These results do not explain the difference in relative toxicities of pyrethrin I and II reported by Chang & Kearns[3] and by workers at Rothamsted.[4, 5] Chang & Kearns[3] do not give the conditions under which they stored their esters, so the relative instability of pyrethrin II might account for its reduced toxicity. However, because they reported only one series of tests, presumably done soon after isolation, the storage experiments reported here indicate that the toxicity of pyrethrin II should have fallen little, if at all, unless stored under extremely unfavourable conditions. Thus regrettably, this work has not resolved the anomaly discussed in the introduction.

Recently, Matsui & Meguro[15] reconstituted pyrethrins I and II from pyrethrolone, isolated by successive fractional distillations, [b.p. 120–140°/0·1–0·5 mm., n_D^{19} 1·5520, $[\alpha]_D^{25}$ + 6·75° (*c*, 10·5, in ethanol)] and found pyrethrin I to be 3 times more toxic than pyrethrin II (Table I). Pure pyrethrolone (from the hydrate) has n_D^{20} 1·5475, $[\alpha]_D^{19}$ + 12·5° (*c*, 13·1 in ethanol)[6] and the alcohol used by Matsui & Meguro therefore contained some isopyrethrolone, n_D^{20} 1·5894 $[\alpha]_D^{20}$ − 5·6° (*c*, 4·82 in ether) produced by thermal isomerisation.[10] Isopyrethrins I and II have low insecticidal activity,[8] so the esters tested by Matsui & Meguro may have been less active than naturally occurring pyrethrins I and II.

Acknowledgments

The authors thank Dr. C. N. Smith and Mr. W. F. Barthel for the flies of the Regular and Cradson P strains, the Statistics Dept., Rothamsted, for calculating the ld–p lines, Mrs. P. Myson for her technical assistance and Mr. A. Bramwell for determining the nuclear magnetic resonance spectra.

Rothamsted Experimental Station
Harpenden
Herts.

Received 4 May, 1964

References

1 Gersdorff, W. A., *J. econ. Ent.*, 1947, **40**, 878
2 Incho, H. H., & Greenberg, H. W., *J. econ. Ent.*, 1952, **45**, 794
3 Chang, S. C., & Kearns, C. W., *J. econ. Ent.*, 1962, **55**, 919
4 Sawicki, R. M., Elliott, M., Gower, J. C., Snarey M., & Thain, E. M., *J. Sci. Fd Agric.*, 1962, **13**, 172
5 Sawicki, R. M., *J. Sci. Fd Agric.*, 1962, **13**, 260
6 Elliott, M., *Chem. & Ind.*, 1958, p. 685
7 Elliott, M., *J. appl. Chem.*, 1961, **11**, 19
8 Elliott, M., *Chem. & Ind.*, 1960, p. 1142
9 Bramwell, A., Crombie, L., Elliott, M., & Janes, N. F., unpublished results
10 Elliott, M., *J. chem. Soc.*, 1964, p. 888
11 Sawicki, R. M., & Green, M., *Bull. ent. Res.*, 1964, in press
12 Sawicki, R. M., & Thain, E. M., *J. Sci. Fd Agric.* 1961, **12**, 137
13 Finney, D. J., 'Probit Analysis', 1952, p. 307 (Cambridge Univ. Press)
14 Sawicki, R. M., *J. Sci. Fd Agric.*, 1962, **13**, 283
15 Matsui, M., & Meguro, H., *Agric. biol. Chem.*, 1964, **28**, 27

STUDIES ON THE PREPARATION, CHEMICAL COMPOSITION AND NUTRITIVE VALUE OF A SPRAY-DRIED SOYA FOOD SUITABLE FOR FEEDING WEANED INFANTS

By S. R. SHURPALEKAR, SOMA KORULA, M. R. CHANDRASEKHARA and M. SWAMINATHAN

A process for the preparation of a spray-dried food (26% protein, 18% fat) based on full-fat soya flour, dextrin–maltose mixture and hydrogenated groundnut oil and fortified with DL-methionine and certain vitamins and minerals has been described. When packed in air in sealed tin containers, the food was organoleptically acceptable at the end of 6 months' storage at 37°. The protein efficiency ratio (PER) of the food was 2·47 ; fortification of the food with DL-methionine at a level of 4 g. per kg. markedly increased its PER to almost the same level as that of a control milk food of similar composition. The overall growth-promoting value of the product, with or without added methionine as also that of 3 : 1 blend of the above foods with cane sugar was significantly higher than that of the milk food or milk food–sugar blend. However, no significant differences were observed in the feed efficiency ratios obtained for soya food (with or without added methionine) and milk food. It may be concluded that in many developing regions where milk is in short supply and soya-bean is readily available, the spray-dried food costing only about 4s. per kg. and possessing an overall nutritive value comparable to that of a milk food of similar composition can be manufactured on large scale and used for supplementary feeding of weaned infants.

Introduction

In view of the acute shortage and high cost of milk in several developing countries of Asia, Africa and Latin America, a considerable amount of work has been carried out by many workers on the development of fortified milk substitutes based on soya-bean and groundnut and suitable for use as supplements to the diets of infants and children.[1–3] Milk substitutes in dried form have the advantages of compactness, good shelf life, low cost and ease of transportation. In Indonesia, a spray-dried milk substitute known as *Saridele* is being manufactured from a blend of soya-bean and groundnut or sesame with UNICEF aid.[3] Spray-dried milk substitutes like Mull-soy, Soya-lac, Sobee etc. are being manufactured in America for feeding infants and children allergic to cow's milk.[4–5] Dean[6] described a process for the preparation of a spray-dried malt food based on barley malt and soya-bean. The present paper describes the results of studies on the preparation, chemical composition and nutritive value of a spray-dried food based on full-fat soya flour, dextrin–maltose mixture and hydrogenated groundnut oil fortified with DL-methionine and certain essential vitamins and minerals.

I. Preparation, chemical composition and shelf-life of the spray-dried soya food

Experimental

Materials

Full-fat soya flour was prepared according to Shurpalekar *et al.*[7] Dextrin–maltose mixture, skim milk powder and hydrogenated groundnut oil used in this study were commercial samples of good quality.

Preparation of spray-dried soya food.—The process described briefly is as follows. A dispersion of full-fat soya flour in 6 times its weight of water, with the pH of the dispersion adjusted to 8·0, was stirred mechanically for 10 min. and homogenised in a triple homogeniser at a pressure of 1000 p.s.i. It was then passed through a 60-mesh vibrating sieve to remove coarse particles and fibre and the pH of the dispersion was readjusted to 6·8. Sufficient amounts of dextrin–maltose mixture and hydrogenated groundnut oil were added to the soya dispersion, so that the fat and protein contents of the final spray-dried product were about 18 and 26% respectively. The dispersion, containing about 22% of total solids, was homogenised, pasteurised and dried in a 'Starcosa' spray-dryer, with the temperature of the inlet air about 120° and that of the outlet air about 80°. The product was fortified by dry-mixing 100 g. of the food with DL-methionine (0·4 g., i.e., 1·6 g./16 g. N), tricalcium phosphate (2 g.), and vitamin A (1500 i.u.),

vitamin D (400 i.u.), vitamin C (30 mg.), thiamine (0·5 mg.), riboflavin (1·0 mg.) and niacin (4·8 mg.), so that 100 g. of the food would provide about three-quarters of the daily requirements of different nutrients of weaned infants. The soya food had a pale cream colour and reconstituted readily in warm water forming a fine dispersion which could be easily fed to weaned infants.

Preparation of milk food.—A milk food having a composition similar to that of spray-dried soya food was prepared by mixing skim milk powder (74 parts), hydrogenated groundnut oil (18 parts) and dextrin–maltose mixture (8 parts) in a Consul mixer for 15 min. The food (100 g.) was fortified with iron pyrophosphate (25 mg.) and vitamin A (1500 i.u.), vitamin D (400 i.u.), vitamin C (30 mg.), thiamine (0·5 mg.), riboflavin (0·5 mg.) and niacin (5 mg.), so that it provided nearly the same amounts of vitamins and minerals as the soya food. This fortified milk food was used as control in the animal experiments.

Methods

The chemical composition of the fortified soya food as compared with that of the fortified milk food was determined by A.O.A.C. methods[8] and the standard plate count according to the methods of American Public Health Association.[9] The shelf-life of the food packed in polythene bags and kept in hermetically sealed tin containers at room temperature (27–29°) and 37° was studied by testing the organoleptic acceptability at monthly intervals.[10]

Results

Table I shows that the chemical composition of the soya food is similar to that of infant milk food specified by the Indian Standards Institution.[11] The total plate count (18,000–22,000 per g. of food) was below the limit laid down by the Indian Standards Institution for infant milk food. The product was free from *Esch. coli*, *Salmonella* and pathogenic anaerobes.

The results of shelf-life studies indicate that the food was organoleptically acceptable after a period of 6 months at 37°. No colour change or development of off-flavours was observed.

II. Nutritive value of the spray-dried soya food

Experimental

Methods

Amino-acid composition.—The essential amino-acid composition of the soya and milk foods was determined according to the method of Krishnamurthy *et al.*[12]

The protein efficiency ratio (PER).—The PER of the soya food was determined at 10% level of protein intake by the rat growth method of Osborne *et al.*[13] Weanling male albino rats (Wistar strain), weighing about 46 g., were grouped according to body weight and litter in a randomised block design. The animals were housed individually in raised wire screen-bottom cages and were fed *ad lib.* on experimental diets, containing 10% of protein from the soya food (with or without added methionine) and the milk food. The diets provided adequate amounts of other nutrients. Records of daily food intake and weekly gain in body weight were maintained for each animal. The data regarding the protein intake, weight gains and PER of the foods are given in Table II.

Overall growth-promoting value.—The overall growth-promoting value of the soya food was assessed by growth studies on albino rats. As reconstituted infant foods are usually sweetened with sugar and fed to infants, a 3 : 1 blend of soya food (with or without added methionine) or milk food and powdered cane sugar were also included in this study. Six groups of freshly weaned albino rats weighing about 45 g. (distributed according to a randomised block design ignoring litters, with 5 males and 5 females in each group) were fed *ad lib.* on foods reconstituted in warm water. Records of food intake and weekly gain in body weight of animals were maintained. At the end of 8 weeks' feeding, the haemoglobin content and red blood cell count of the blood (drawn from tail veins), as also the moisture, fat and protein contents of the livers of different groups of animals were determined according to methods used by Shurpalekar *et al*[14] The feed efficiency ratio (FER), i.e., the weight gain per g. of food intake, was calculated.

Table I

Chemical composition of spray-dried soya food

Constituents	Soya food[a]	Milk food[b]
Moisture, g.	2·9	2·9
Protein[c] g.	26·8	26·2
Fat, g.	17·8	18·5
Ash, g.	5·7	5·4
Carbohydrates, g. (by diff.)	46·8	47·0
Calcium, g.	0·92	0·98
Phosphorus, g.	0·82	0·85
Iron, mg.	6·2	6·0
Vitamin A, i.u.	1450	1450
Vitamin D, i.u.[d]	400	400
Vitamin C, mg.	30·2	29·5
Thiamine, mg.	0·9	0·8
Riboflavin, mg.	1·4	1·4
Niacin, mg.	5·7	6·2

[a] Fortified with calcium phosphate, thiamine, riboflavin, niacin and vitamins A, D and C
[b] Fortified with iron pyrophosphate, thiamine, riboflavin, niacin and vitamins A, D and C
[c] Obtained by multiplying nitrogen content by 6·25 for soya food and 6·38 for milk food
[d] Added values

Table II

Protein efficiency ratio[1] of the spray-dried soya food

Group	Source of protein in the diet[2]	Initial body weight, g.	Weight gain, g.	Protein intake, g.	P.E.R.
A	Soya food	46·4	78·6	31·7	2·47
B	Soya food + methionine	46·5	92·7	31·7	2·92
C	Milk food	46·6	76·0	25·5	2·99
					±0·05 (14 df)
Critical difference (one-tailed test) at	5% level				0·12
	1% level				0·17
	0·1% level				0·25

[1] Mean values for 8 males per group; duration of experiment 4 weeks
[2] Level of protein 10% (on moisture-free basis)

Results

Amino-acid composition and protein efficiency ratio (PER)

The results presented in Table III indicate that the soya flour is deficient only in methionine. This deficiency was made up by fortification with DL-methionine at a level of 4 g. per kg. of food. The results presented in Table II indicate that the PER of soya food (2·47) was significantly lower (P <0·001) than that (2·99) of the milk food. Fortification of the soya food with DL-methionine improved markedly the PER to 2·92, a value nearly equal to that of milk food.

Overall growth-promoting value

Growth and feed efficiency ratio (FER).—The soya food with or without added methionine, as also their 3 : 1 blends with sugar, promoted significantly higher (P <0·001) growth (156–180 g./8 weeks) in rats than the milk food or milk food–sugar blend (113–130 g./8 weeks). The growth observed in rats receiving the soya and milk foods was significantly lower (P <0·05–P <0·01) than the corresponding values obtained for 3 : 1 blends of the above foods with sugar.

Table III

Essential amino-acid composition of spray-dried soya food

Amino-acid, g./16 g. N	Soya food	Soya food + methionine	Milk food	F.A.O. reference protein pattern
Arginine	7·1	7·1	3·8	—
Cystine	1·6	1·6	0·8	—
Histidine	2·3	2·3	2·8	—
Isoleucine	5·3	5·3	6·6	4·2
Leucine	7·8	7·8	10·2	4·8
Lysine	6·4	6·4	8·0	4·2
Methionine	1·3	2·9	2·8	2·2
Total S-amino-acids	2·9	4·5	3·6	4·2
Phenylalanine	5·0	5·0	5·2	2·8
Threonine	3·8	3·8	4·5	2·8
Tryptophan	1·2	1·2	1·4	1·4
Valine	5·2	5·2	6·9	4·2

Table IV

Growth and composition of blood and liver of rats fed on the spray-dried soya food[1]

Group	Diet	Weight gain[2] g./8 weeks	F.E.R.[3, 4]	Red blood cells[3] 10^6/cu. mm.	Haemo-globin[3] g./100 ml.	Composition of liver[3] Moisture, %	Fat,[5] %	Protein,[5] %
A	Soya food (S.F.)	156·2	0·284	8·4	16·0	69·0	3·6	20·4
B	S.F. (3 parts) + sugar (1 part)	170·4	0·286	8·4	16·0	69·7	3·6	20·0
C	S.F. + methionine	171·8	0·298	8·3	15·7	68·8	3·8	20·6
D	S.F. (3 parts) + methionine + sugar (1 part)	179·5	0·296	8·5	15·8	69·1	3·7	20·4
E	Milk food	113·4	0·298	7·7	15·2	69·7	3·7	20·3
F	Milk food (3 parts) + sugar (1 part)	129·6	0·307	8·0	15·7	69·4	3·7	20·4
	Standard error of the mean	±4·58 (40 df)	±0·007 (20 df)	±0·12 (20 df)	±0·23 (20 df)	±0·28 (20 df)	±0·08 (20 df)	±0·40 (20 df)
	Critical difference (one-tailed test) at :							
	5% level	10·9	0·016	0·3	0·6	0·69	—	—
	1% level	15·7	0·023	0·5	0·8	—	—	—
	0·1% level	21·4	0·033	0·6	1·2	—	—	—

[1] Duration of experiment—8 weeks
[2] Mean values for 5 males and 5 females (of initial body weight 45 g.) per group
[3] Mean values for 5 males (of initial body weight 47 g.) per group
[4] Feed efficiency ratio—weight gain/food intake
[5] None of the differences are significant

The FER (0·286) of a 3 : 1 blend of soya food (without added methionine) and sugar was significantly lower ($P < 0·05$) than that (0·307) of milk food–sugar blend. However, no significant differences were observed in the FER values (0·284–0·298) obtained for soya food (with or without added methionine) and milk food.

Composition of blood and liver.—The red blood cell count of the soya food, with or without added methionine or sugar was significantly higher than the corresponding values obtained for milk food, with or without added sugar. The haemoglobin levels in the blood of rats fed on soya food with or without added methionine were significantly higher than those obtained for rats receiving the milk food ; however, the values obtained for 3 : 1 blends of different foods with sugar did not differ significantly. No significant differences were observed in the fat and protein contents of the livers of rats fed on different foods with or without added methionine or sugar.

Discussion

The results of the present investigation have shown the possibilities of preparing a highly nutritious milk substitute based on full-fat soya flour, dextrin–maltose mixture and hydrogenated groundnut oil and fortified with the limiting amino-acid—DL-methionine—and certain essential vitamins and minerals. The spray-dried soya food containing 26% of protein and 18% of fat can be reconstituted easily in warm water and after sweetening with sugar, can be fed to infants aged 6 months or more. The food when fed at a level of 100 g./day would provide about three-quarters of the daily requirements of different nutrients of weaned infants. The lower overall growth-promoting value of milk food may be attributed to its high lactose content which is not well tolerated by rats.[15] The growth-promoting value of 3 : 1 blend of soya food and sugar containing about 19% of protein (the form in which such foods are generally fed to infants) was somewhat higher than that of a milk food–sugar blend of similar composition. The ex-factory cost of the product packed in polythene bags and repacked in 4-gal. tin containers is expected to be about 4s. per kg. as compared with 8s. per kg. of an infant milk food. It may be concluded from the results that, in many developing countries where milk is in short supply and soya-bean is readily available, spray-dried soya food fortified with DL-methionine and certain vitamins and minerals and possessing an overall nutritive value comparable to that of a milk food of similar composition, can be manufactured on a large scale and used for the supplementary feeding of weaned infants.

Acknowledgment

The authors thank Miss D. Rajalakshmi and Mr. A. N. Sankaran for help in statistical analysis of results.

Central Food Technological Research Institute
Mysore
India

Received 22 June, 1964

References

[1] Food & Agriculture Organisation, 'Year Book of Food and Agricultural Statistics', 1962, Vol. 16 (Rome: F.A.O.)
[2] *Indian Coun. med. Res. Spec. Rep. Ser.*, 1955, No. 31 (New Delhi: The Council)
[3] 'Report of the FAO/UNICEF Regional School Feeding Seminar for Asia and the Far East', 1959, *Nutr. Mts Rep. Ser.*, 1959, No. 22 (Rome: F.A.O.)
[4] Meyer, H. F., 'Infant Foods and Feeding Practice', 1960 (Illinois: C. C. Thomas)
[5] Cahill, W. M., Schroeder, L. J., & Smith, A. H., *J. Nutr.*, 1944, **28**, 209
[6] Dean, R. F. A., 'Plant Proteins in Child Feeding', *Med. Res. Coun. Spec. Rep. Ser.*, 1953, No. 279 (London: The Council)
[7] Shurpalekar, S. R., Chandrasekhara, M. R., Swaminathan, M., Sreenivasan, A., & Subrahmanyan, V., *J. Sci. Fd Agric.*, 1964, **15**, 370
[8] 'Official and Tentative Methods of Analysis', 9th edn, 1960 (Washington, D.C.: Ass. off. agric. Chem.)
[9] American Public Health Ass., 'Standard Methods for the Examination of Dairy Products', 11th edn, 1960 (New York: The Association)
[10] Chandrasekhara, M. R., Sreenivasamurthy, V., Swaminathan, M., Bhatia, D. S., & Subrahmanyan, V., *Food Sci., Mysore*, 1957, **6**, 228
[11] Indian Standards Specifications for Infant Milk Foods, I.S.: 1547, 1960 (New Delhi: Indian Stand. Instn)
[12] Krishnamurthy, K., Tasker, P. K., Ramakrishnan, T. N., Rajagopalan, R., & Swaminathan, M., *Ann. Biochem. exp. Med.*, 1960, **20**, 73
[13] Osborne, T. B., Mendel, L. B., & Ferry, E. L., *J. biol. Chem.*, 1919, **37**, 223
[14] Shurpalekar, S. R., Chandrasekhara, M. R., Lahiry, N. L., Swaminathan, M., Indiramma, K., & Subrahmanyan, V., *Ann. Biochem. exp. Med.*, 1960, **20**, 145
[15] Riggs, L. K., Beaty, Annabel, & Mallan, B., *J. agric. Fd Chem.*, 1955, **3**, 333

SPECIFIC AND VARIETAL DIFFERENCES IN SODIUM AND POTASSIUM IN GRASSES

By G. ap GRIFFITH, D. I. H. JONES and R. J. K. WALTERS

Marked differences in sodium content between and within species were maintained in primary and recovery growth throughout the growing season. There was a general, though rather loose inverse relationship between Na and K content.

The species studied (in descending order of Na) were: *Lolium perenne, Dactylis glomerata, L. multiflorum, Festuca arundinacea, F. pratensis, Phleum pratense.*

Nitrogen raised the Na content whether applied as nitrate of soda, sulphate of ammonia or Nitro-Chalk, but the effect was much less marked in *P. pratense.*

Introduction

Collander,[1] studying the selective absorption of cations by higher plants, found that the sodium content showed a variability equalled only by manganese. Grasses were not included in Collander's study nor in the later work of Harmer *et al.*,[2] who reported that the species studied ranged from those which gave no yield response to Na when K was in short supply to those which gave a response when K was in ample supply.

Lehr[3] quoted several workers who have reported a wide range in the Na content of grassland herbage. Their observations were not related to species, but Lehr compared different grass species and classified them in the following descending order of their capacity to absorb Na:

Lolium perenne, Dactylis glomerata, Poa trivialis, Festuca pratensis, Phleum pratense, Poa pratensis. Garaudaux[4] also has stated that *Dactylis* and *Festuca* have high and low Na contents respectively.

The present authors have recently examined the Na and K content of grass species and varieties grown in four series of trials. These were sited close together on a level field at an elevation of 80 ft., less than 2 miles from the west coast of Wales; the soil was light loam of the Teifi series, pH 6·2, with exchangeable cations (mequiv. %) of the order Ca, 7–14, K, 0·2–0·4, Na, 0·2–0·4.

Experimental and results

Series 1

Material was taken from replicated pure swards composed of the following: *Lolium multiflorum* S.22; *L. perenne* S.23, S.24; *Dactylis glomerata* S.37; *Festuca arundinacea* S,170; *F. pratensis* S.215 and *Phleum pratense* S.48. All plots had been treated with 3 cwt./acre of compound fertiliser (12 : 12 : 18) plus 3 cwt./acre of 15% Nitro-Chalk early in March 1962. Eight samples of primary growth were taken at intervals from 20 March to 22 June in the first harvest year.

The Na and K contents (Table I) showed essentially similar seasonal patterns of initial rise followed by a decline. The differences in K between the grasses were quite marked, but for Na they were generally relatively greater, more distinct, and better maintained throughout the sampling period.

Table I

The sodium (Na) and potassium (K) of eight grasses

mequiv., % of dry matter

(Series 1. Primary growth)

	March 20		April 10		April 24		May 10		May 18		May 28		June 6		June 22		Average	
	Na	K	Na	K	Na	K	Na	K	Na	K	Na	K	Na	K	Na	K	Na	K
Lolium perenne S.24	9·7	44·9	15·5	61·3	14·7	54·4	14·1	51·5	13·5	46·7	11·0	42·6	9·1	37·2	5·7	29·8	11·7	46·0
L. perenne S.23	7·8	49·4	9·1	65·1	9·9	57·4	9·5	58·3	8·7	58·7	7·8	48·2	7·4	42·7	5·5	41·7	8·2	52·7
Dactylis glomerata S.37	10·1	60·5	8·9	76·2	9·4	73·6	11·9	74·9	13·1	67·9	12·9	57·7	10·1	53·9	9·4	48·2	10·7	64·1
L. multiflorum S.22	4·5	52·8	6·0	73·6	5·8	62·1	5·5	57·7	5·2	55·4	3·4	42·1	3·9	32·8	2·6	31·6	4·5	50·0
Festuca arundinacea S.170	4·3	52·6	6·7	78·2	4·7	71·0	3·9	74·1	4·6	60·5	3·9	52·6	3·1	48·2	2·7	42·5	4·2	59·9
F. pratensis S.215	2·7	60·5	3·3	72·3	1·8	68·7	2·0	64·6	1·6	54·7	1·3	47·0	1·1	44·4	1·6	39·7	1·9	56·5
Phleum pratense S.48	*	*	2·4	65·7	1·7	61·5	1·3	63·7	1·1	58·5	1·2	49·7	0·8	49·1	0·9	55·9	1·3	57·7

*Insufficient growth to provide a sample

Within species there is an indication of an inverse relationship between Na and K. Thus of the two *Lolium perenne* varieties, the higher in Na at all sampling dates is the lower in K.

The grasses in descending order of Na content are *L. perenne* S.24, *D. glomerata* S.37. *L. perenne* S.23, *L. multiflorum* S.22, *F. arundinacea* S.170, *F. pratensis* S.215, *P. pratense* S.48, This order is essentially similar to that for the species Lehr[3] included in his study.

Series 2

This material was from a replicated sward in which *L. perenne* S.24 was treated with Nitro-Chalk at four levels of 1, 2, 4 and 8 cwt./acre applied late in February. A basal dressing of 3 cwt./acre of compound fertiliser (0 : 14 : 28) was previously given. Samples of primary growth were taken from 26 March to 13 June in the first harvest year.

The marked effect of the dressings of Nitro-Chalk on the Na content was maintained throughout the period of the trial (Fig. 1). The K content was little affected except at the highest N level. The seasonal pattern of the two elements was also different, K reaching its maximum at the third sampling date, whereas Na was at its peak a month later at the fifth date. The increase in Na due to N was quantitatively greater than the increase in K.

Series 3

One first growth and six subsequent regrowth cuts were taken during April to October in the first harvest year of *L. perenne* S.23 and S.24; *D. glomerata* S.143 and *P. pratense* S.48 which had been treated with heavy dressings of nitrogen applied either as sulphate of ammonia

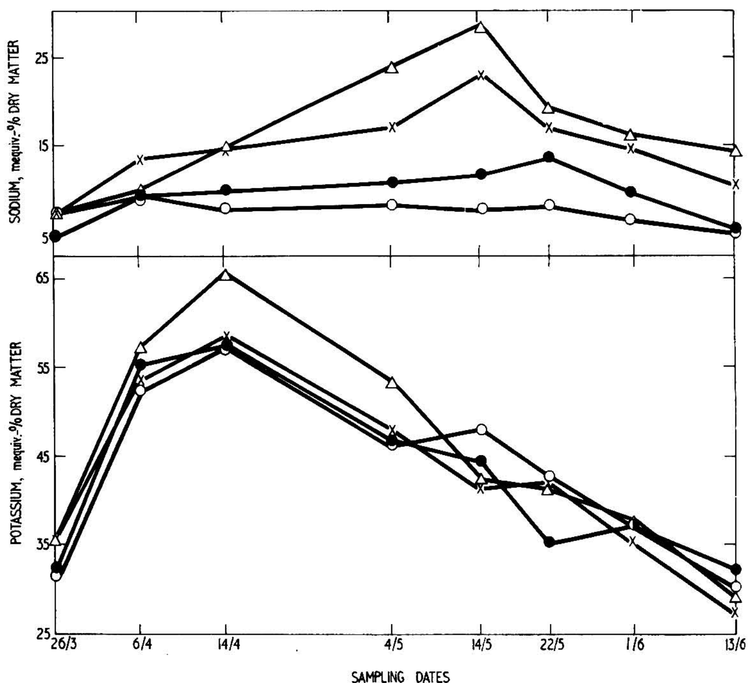

FIG. 1.—*Sodium and potassium content of* Lolium perenne *S.24 at four levels of nitrogen applied as Nitro-Chalk*
Series 2. Primary growth. Sampled 26 March to 13 June
Nitro-Chalk ○ 1 ● 2 × 4 △ 8 cwt./acre

or nitrate of soda; 80 lb. of N was applied after each cut. All plots had been given a basal dressing of 7 cwt./acre of compound fertiliser 0 : 14 : 28.

The effect of the different forms of N-fertiliser on the *Lolium* and *Dactylis* spp. was similar, so the data for *L. perenne* S.24 have been selected as representative for presentation in Fig. 2, together with the data for *P. pratense* S.48. As in Series 2, the N-fertiliser increased the Na content of *L. perenne* S.24, the effect being more marked with each successive cut. As

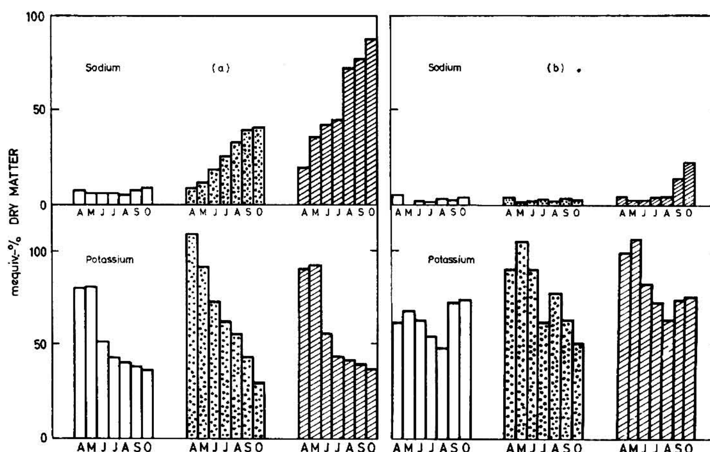

FIG. 2.—*Sodium and potassium content of (a)* Lolium perenne *S.24 and (b)* Phleum pratense *S.48 after treatment with sulphate of ammonia and nitrate of soda*
Series 3. First growth and six subsequent regrowth cuts taken April to October 1962
□ control ⁝⁝⁝ sulphate of ammonia ▨ nitrate of soda

might be expected, the nitrate of soda gave a greater increase in Na than did the sulphate of ammonia. The Na content of *P. pratense*, a low-Na grass, showed little effect from the application of sulphate of ammonia and even the nitrate of soda was relatively unsuccessful in increasing the Na content, thus suggesting a physiological barrier to sodium.

Over the season there is a close inverse relationship between Na and K in the *L. perenne.*

Series 4

The fourth series of samples was from unreplicated observation drills of varieties of *L. perenne* (25), *D. glomerata* (17), *F. pratensis* (16) and *F. arundinacea* (6). Four cuts were taken, one of first growth and three of recovery growth.

There were wide differences in Na and K contents between and within species which were well maintained between cuts (Table II).

Table II

Sodium and potassium in varieties of four grass species (mequiv. % of dry matter)
(Means for four harvests)

	Na	K
Lolium perenne (25 varieties)		
Hunsballe Late III	38	24
Melle Pasture	38	36
S.23	36	45
Vertas Pasture Poly (tetraploid)	35	44
C.B. Pasture Tetraploid (tetraploid)	35	38
Øtofte Dux Late III	34	27
Pelo Pasture	34	43
Ba 6280	33	43
S.24	33	36
E.F. Trifolium Late III	32	33
Pajbjerg Lenta Late III	32	29
Vertas Hybrid Long Rotation	31	41
Viris Late	31	37
S.101	30	45
Doublet Hay	30	37
N. Irish	29	42
Øtofte Early III	26	45
Trifolium T.10	25	48
Vertas Hay Poly (tetraploid)	23	51
C.A.570	21	40
Pajbjerg Presto Early III	19	47
S.321	17	51
Trifolium T.11	17	49
Pajbjerg Verna Early III	14	49
Norlea	10	56
Mean	29	41

	Na	K
Festuca pratensis (16 varieties)		
Vertas Pasture New	8	45
C.B. Pasture	5	49
Trifolium II	4	44
Hinderupgaard II	3	50
Mommersteegs Pasture	3	47
Barenza Pasture	3	49
Sceempter Pasture	3	48
Pajbjerg II	2	51
Øtofte Early II	2	41
Vertas Hay New	2	44
Mommersteegs Hay	2	49
Barenza Hay	2	49
S.215	2	46
C.B. Hay	2	45
Sceempter Hay	2	52
S.53	2	48
Mean	3	47

	Na	K
F. arundinacea (6 varieties)		
Bn 276	30	31
Bn 232	24	35
Bn 294	24	39
Alta	23	39
Bn 286	23	48
S.170	17	62
Mean	23	42

	Na	K
Dactylis glomerata (17 varieties)		
S.143	44	27
Trifolium Late	44	32
S.37	44	32
S.26	40	29
C.B.	37	37
S.345	35	38
Dorise	35	48
Mommersteegs Early	33	39
Barenza	33	36
Trifolium A 11	30	43
Vertas Hay-Pasture New	26	52
Roskilde III	26	40
Trifolium E.11	26	46
Potomac	21	42
Sceempter Smalbladige	21	53
Tammisto	11	49
Latar	5	47
Mean	30	41

The average Na content and range of varietal variation was similar for *L. perenne* and *D. glomerata*, although in the Latar variety of *D. glomerata* the content was exceptionally low. *F. arundinacea* was slightly lower in Na content. The markedly lower levels of Na in *F. pratensis* compared with those in the other species confirm the results in Series 1 (Table I).

Discussion

It is clear that Na shows a wide variation between grass species and also between varieties of the same species. In the four series, the species shown to be low in Na were *P. pratense* and *F. pratensis*, as already reported by Lehr.[3]

Nitrogenous fertilisers applied as Nitro-Chalk, sulphate of ammonia or nitrate of soda increased the Na content of *L. perenne* and *D. glomerata* but had little effect on *P. pratense*. Lehr[3] also found that N-manuring raised the Na content, but, contrary to the present finding, claimed that this only resulted from the use of nitrate of soda.

This effect of nitrogen on Na may be due to K being present in insufficient supply to cope with the increased growth resulting from the application of nitrogenous fertilisers. If this is so it would seem at least that Na is able to replace K in providing the cation requirements for increased growth.

Whether grasses respond to Na applications does not seem to have been investigated. Harmer & Benne,[5] in a study which did not include grasses, stated that in species normally low in Na there will be no response to added Na, whereas species with a high Na content would make a response probable. None of the four series discussed in this paper gave a clear indication of the natural level of Na in the grasses studied, but it may be seen that some species take up Na more readily than others, and the implication of this on the manurial side needs investigation.

It has been suggested[6] that a feed for a lactating animal should contain more than 0·15% of Na. In present observations the sodium in *P. pratense* and *F. pratensis* failed to reach this level. The implications of this need further study, although in practice in a mixed sward there may be an adequate contribution from other species. A shortage of Na is perhaps unlikely to be serious when animals are allowed access to salt, but free-ranging animals on the hills may need separate consideration, and hill species of grasses and other herbage should be examined.

A high K content in grass has been repeatedly suggested as a contributory cause of hypomagnesaemia in ruminants, possibly through its association with a low magnesium. More recently Na has been implicated, and Butler[7] has shown a close agreement between low Na content in pastures and increased incidence of grass tetany. Since there is a general inverse relationship between K and Na this might be expected to follow, but in fact Butler's results indicate a more consistent relationship of grass tetany with Na than with K and show, as in our own results, that a low herbage Na is not always accompanied by a high K.

Welsh Plant Breeding Station
Aberystwyth

Received 26 May, 1964; amended manuscript 21 July, 1964

References

1 Collander, R., *Plant Physiol.*, 1941, **16**, 691
2 Harmer, P. M., Benne, E. J., Laughlin, W. M., & Key, C., *Soil Sci.*, 1953, **76**, 1
3 Lehr, J. J., *Proc. 8th Int. Grassl. Congr. 1960*, 1961, p. 101
4 Garaudaux, J., *Rep. World Congr. agric. Res* (Rome), 1959, p. 593
5 Harmer, P. H., & Benne, E. J., *J. Amer. Soc. Agron.*, 1941, **33**, 952
6 Russell, F. C., & Duncan, D. L., 'Minerals in Pastures', *Tech. Commun. Commw. Bur. Anim. Nutr.*, 1956, No. 15, 133
7 Butler, E. J., *J. agric. Sci.*, 1963, **60**, 329

LIPIDS AND PROTEIN DENATURATION IN FISH MUSCLE

By JUNE OLLEY* and W. R. H. DUNCAN†

Gas–liquid chromatography has been used to determine the composition of the neutral lipids and phospholipids of the musculature of several species of fish. The nature of the free fatty acids (FFA) developed during cold storage of these species has also been examined. The phospholipids of three teleosts and an elasmobranch were similar containing 50–60% C_{20} + C_{22} polyenes.

An attempt has been made to correlate the rate of protein denaturation in these species with the nature of the FFA produced during cold storage. There were no immediately obvious trends either in the nature of the FFA produced or in the composition of the phospholipids or neutral lipids from which the FFA derived. However, species with higher percentages of C_{22} hexaenes in the FFA liberated during frozen storage did on the whole show greater rates of protein denaturation.

Introduction

The current interest in the theory that the production of free fatty acid (FFA) in frozen fish muscle may cause the denaturation of the structural proteins of the myofibrils was shown by the lively discussion of the topic at the FAO ' Fish in Nutrition ' conference.[1] This has been followed by two subsequent papers[2, 3] at the FAO symposium on the ' Significance of Fundamental Research in the Utilisation of Fish '. The theory was supported by the earlier work of Dyer & Fraser[4] and the work of Olley & Lovern[5] on frozen cod. However, when Olley *et al.*[6] extended the theory to other species they found that three teleosts, cod, halibut and lemon sole, produced very similar amounts of FFA on cold storage at −14°, yet had completely dissimilar protein denaturation rates as measured by the solubility of actomyosin in 5% sodium chloride. At this time King *et al.*[7] found that addition of linoleic and linolenic acid to actomyosin solutions caused insolubilisation of the protein, the initial amount required per mg. of protein-N being less for the latter acid, but the subsequent rate of reaction being greater with linoleic acid. Since this preliminary communication Anderson & Steinberg[8] have found that when the experiments are repeated with fatty acid soaps, rather than the free acid, the same amount of actomyosin is denatured with acids ranging from $C_{18}{}^{=2}$ to $C_{22}{}^{=6}$. Storage of the actomyosin solutions in the presence of the soaps produced a different rate of insolubilisation for each acid soap, but there was no rationale in the results; the rate could not be related to unsaturation or chain length of the soaps.

Anderson & Steinberg[9] have also very recently found that a critical level of sodium linolenate had to be added to the buffered salt solutions used to extract actomyosin from various species of fish before insolubilisation commenced. The amount of soap required to initiate ' denaturation ' increased with increasing lipid content of the flesh of the fish within the limited range 0·8–1·1 g. lipid per 100 g. of fish muscle. Hanson & Olley[2] calculated from Anderson & Steinberg's data that approximately 100 mg. of neutral lipid would protect actomyosin from insolubilisation by 200 mg. of FFA. Lovern[10] pointed out that the only lipid measurement which rated the three teleosts cod, halibut and lemon sole, in the same order as their rates of protein denaturation during frozen storage was the *percentage* of FFA in the total lipid. As the average content of lipid in the three species was 0·67%, 0·76% and 0·85% respectively,[6] the small differences in percentage FFA caused by similar amounts of FFA and different quantities of total lipid, were not considered significant. The recent model experiments of Anderson & Steinberg[9] have shown that very small quantities of neutral lipid can be extremely important in reducing the amount of actomyosin insolubilised by FFA. Hanson & Olley[2] have produced evidence to indicate that neutral lipid protects protein from FFA denaturation *in situ* and not just at the homogenisation stage of the soluble protein determination.

The amount of neutral lipid in proportion to the free fatty acid liberated during cold storage may be sufficient to explain some of the apparent differences in protein ' denaturation ' in

* Torry Research Station (D.S.I.R.), Aberdeen
† Rowett Research Institute, Bucksburn, Aberdeen

various species of fish as measured by the salt solubility test. However, in the interim period results have been obtained by working on the original suggestion of King *et al.*[7] that the nature of the free fatty acid produced during cold storage could influence the rate of actomyosin denaturation. Analyses by gas–liquid chromatography of the free fatty acid, and the phospholipids and/or neutral lipids from which they derived have been done on the four species of fish examined in the previous paper (Olley *et al.*[6]).

Experimental

Preparation of methyl esters

Four specimens each of sole, halibut and piked dogfish and six cod were used. They were gutted and iced after catching and had not been more than 4—5 days in melting ice. Two fish of each species were skinned and filleted, the fillets minced and the lipid extracted from 100 g. of muscle as described previously.[6] The other fish were frozen in an air-blast at −29°, glazed with water and stored at −14° for 11½ weeks. The two extra cod were stored similarly for 26 weeks. The fish were thawed overnight in a chill room at 4°, filleted, skinned and the lipids extracted from 100 g. of the minced tissue. The neutral lipids were separated by silicic acid chromatography[5] from the phospholipids, the latter being eluted with 1 : 10 chloroform/methanol. The FFA in the ether solution of the neutral lipids from fresh cod and cod stored for 26 weeks at −14° was washed out with aqueous 2% sodium carbonate. FFA from fish stored for 11½ weeks at −14° were separated by the method of McCarthy & Duthie[11] on the whole lipid extract. The ether solutions of FFA obtained by this method were washed with water to remove all formic acid and the FFA methylated after evaporation of the ether.

Phospholipids, neutral lipids and FFA were methylated with 5% methanolic sulphuric acid. Methyl esters were dried overnight in a desiccator containing phosphorus pentoxide.

The FFA in fresh fish of the species studied did not amount to more than 1–2%[5,6] of the total lipid and this was included with the neutral lipid fatty acids. The sole and halibut neutral lipids were methylated without saponification and the non-saponifiable material removed by passage of the methyl esters in light petroleum containing 1% ethyl ether through a silicic acid column. The dogfish neutral lipids were saponified for 2 h. with 0·5N ethanolic potassium hydroxide under reflux, non-saponifiable matter was removed and the fatty acids methylated. All phospholipids were methylated directly without saponification.

In view of the fact that the unsaturation of the dogfish body oil was higher than expected, the liver oil of this fish was examined. The experimental procedure for the liver oil was the same as for the body neutral lipids.

Experiments were repeated when the methyl ester analysis seemed unexpected or of particular interest. The results therefore refer to fish caught at different times of the year and the month of catching has been recorded. The FFA results should where possible be compared with the analyses of the methyl esters of the fatty acids from phospholipids or neutral lipids of fish caught in the same month.

On completion of the first series of experiments it appeared that there might be some correlation between protein denaturation and the percentage of C_{22} polyunsaturated acids in the free fatty acids liberated during cold storage and so a further species (haddock) was included in this study. Six haddock were therefore frozen and stored at −14° for 11·5 weeks and then treated in two batches of three fish. The FFA were isolated and methylated as described for the other species for 11·5 weeks.

Gas–liquid chromatography of methyl esters

Fatty acid methyl esters were analysed by gas–liquid chromatography using a Pye Argon Chromatograph (W. G. Pye & Co. Ltd., Cambridge) containing a ^{90}Sr detector. The standard glass columns contained ' Embacel ' (60–100 mesh acid-washed kieselguhr, May & Baker Ltd., Dagenham) impregnated with 20% by weight of polymerised ethylene glycol succinate (prepared as described by Farquhar *et al.*[12] for the corresponding adipate) or ' Embacel' which had been pre-treated with dimethyldichlorosilane according to Silk & Hahn[13] and coated with 0·7% by

weight of Apiezon L grease (Shell Chemicals Ltd., London). Column temperatures were in the range 168–183° and the argon flow was usually 50 c.c./min.

Each sample of esters, before and after hydrogenation (Popják & Tietz[14]), was chromatographed on each of the two liquid phases. Measurement of peak areas on the chromatograms by the triangulation procedure gave the percentage by weight of each component. Identity of each peak was established from a knowledge of the relative retention volumes of the esters and authentic esters on each of the two liquid phases. Authentic esters included methyl eicosapentaenoate and methyl docosahexaenoate (kindly supplied through the courtesy of Dr. W. M. Goldwater, National Institutes of Health, U.S.A.).

Results

The detailed fatty acid analyses are shown in Table I. The total phospholipids of all four species were similar and had 50–60% of C_{20} + C_{22} acids which were mostly pentaenes and hexaenes. The sole phospholipids and neutral lipids were characterised by a higher percentage of C_{22} pentaenes and a lower percentage of C_{22} hexaenes than the other three species. The C_{22} acids were always considerably in excess of the C_{20} acids in the phospholipids, a pattern which appears to be typical of fish muscle tissue.[15, 16] Richardson *et al.*[17, 18] have found that the fatty acids of the heart muscle mitochrondria of several species of fish contain a characteristically higher proportion of C_{22} hexaenes than of C_{20} pentaenes, while the fatty acids of the corresponding liver mitochrondria do not.

The neutral lipid analysis of the body oil of the halibut caught in December agrees well with the results quoted by Lovern[19] but the neutral lipids of the halibut caught in July were far more unsaturated. The neutral lipid analysis of the body oil of the sole was in reasonable agreement with the detailed analysis of Klenk & Eberhagen[20] for sole liver.

The neutral lipids of both the body and liver oils of the dogfish caught in December were more unsaturated than the liver oils studied by Guha *et al.*[21] and also far more unsaturated than both the liver and body oils of dogfish caught in December in Puget Sound (Malins *et al.*[22]). The liver oils studied by these workers contained 2·3% $C_{20}{}^{=5}$ acids and 5·7% $C_{22}{}^{=5}$ and $C_{22}{}^{=6}$ acids, while the body oils contained 6·3% $C_{20}{}^{=5}$ acids and 14% $C_{22}{}^{=5}$ and $C_{22}{}^{=6}$ acids. We are in agreement with these workers in that the dogfish flesh oils in the present study were almost twice as unsaturated as the liver oils. However, their work showed that in the flesh it was mainly fatty acids from the diacylglyceryl ethers which were contributing to the high unsaturation. It is impossible to tell from the analyses of the dogfish FFA liberated after 11·5 weeks at −14° whether they were derived from neutral lipids or diacylglycerol ethers.

The palmitic/stearic acid ratio was always higher in the FFA of the three teleosts than in the parent phospholipids. Shuster *et al.*[23] have pointed out that a high palmitic/stearic acid ratio is characteristic of neutral lipids and lecithins in a tissue and a low ratio is characteristic of cephalins. In the case of the dogfish where the FFA appear to derive from neutral lipids,[6] the ratio was the same for neutral lipids and FFA. The increased ratio in the teleosts would indicate a preferential hydrolysis of lecithins. Bligh[24] has noted that the acidic cephalins of cod muscle are not hydrolysed during cold storage. Lovern & Olley[25] have confirmed this but point out that when phospholipid hydrolysis ceases, there are still appreciable quantities of lecithin and phosphatidyl ethanolamine unhydrolysed. The present data might indicate that these phospholipids have an unsuitable composition for further enzyme hydrolysis, for example the palmitic/stearic acid ratio of the phospholipids of cod remaining after 26 weeks of storage of the fish at −14° was only 1·6. Alternatively phospholipids of low palmitic/stearic acid ratio may be localised in a particular cell organelle.

Discussion

Lipid hydrolysis and protein denaturation

Synthetic detergents have been known since 1939[26] to denature proteins and there are several examples in the literature of the combination of fatty acids with protein.[27, 28] Electrostatic[3] and also non-polar forces from the long hydrocarbon chains of the detergent or fatty acid are thought to be important[26, 27] and therefore chain length of the molecule is of significance.

Table I

Composition of fish lipid fractions

(Methyl esters % by wt.)

Fish[a]	Treatment	Source of methyl esters	Month caught	C_{14} S[b]	C_{15} S	C_{16} S	C_{16} −2H[c]	C_{17}	Not identified	C_{18} S	C_{18} −2H	C_{18} −4H	C_{18} −6H	C_{18} −8H	C_{19}	C_{20} S	C_{20} −2H	C_{20} −8H	C_{20} −10H	C_{21}	C_{22} −2H	C_{22} −6H	C_{22} −8H	C_{22} −10H	C_{22} −12H	$C_{>22}$	P/S
Cod	Fresh	FFA	March	1·0	0·5	27·2	3·4	tr.	1·8	5·9	12·7	1·4	tr.	0·8	—	—	1·8	2·7	19·5	—	tr.	—	—	0·8	20·5	—	4·6
	,,	Phospholipid	,,	0·5	0·4	20·6	1·6	0·5	—	4·2	10·1	0·8	tr.	0·2	—	—	1·5	2·9	14·6	—	tr.	2·8	1·6	1·2	35·4	1·1	4·9
	11·5 weeks, −14°	FFA	,,	0·6	0·3	23·5	2·4	0·8	0·6	2·9	11·3	0·6	0·3	0·2	0·2	tr.	1·0	5·0	15·7	0·5	tr.	—	tr.	2·5	30·6	1·0	8·1
	26 weeks, −14°	FFA	,,	0·7	0·3	26·3	1·6	—	0·7	3·6	10·0	0·7	tr.	0·5	—	tr.	1·4	2·2	16·5	—	tr.	—	tr.	1·1	34·4	—	7·3
	,, ,,	Phospholipid	,,	0·4	0·8	13·6	1·9	tr.	0·7	8·4	10·2	0·6	tr.	tr.	—	tr.	1·3	3·4	12·2	—	tr.	3·9	—	tr.	42·6	—	1·6
Sole	Fresh	Neutral lipid	(1) June	4·3	0·7	16·5	14·4	1·4	5·5	2·4	12·2	0·3	2·0	1·6	0·7	tr.	3·9	4·0	11·9	0·6	tr.	—	tr.	10·6	7·0	—	6·9
	,,	,, ,,	(2) Dec.	3·3	1·1	20·0	4·7	2·9	3·7	2·2	18·1	1·0	—	0·5	0·3	0·6	6·6	1·6	17·5	0·3	0·5	—	2·1	6·4	6·6	—	9·0
	,,	Phospholipid	(1) June	1·0	0·4	20·3	2·9	0·7	2·6	6·1	9·3	—	—	—	0·2	—	tr.	6·4	17·3	0·2	tr.	—	2·1	9·3	21·2	—	3·3
	11·5 weeks, −14°	FFA	(3) Feb.	1·6	0·7	38·5	4·0	0·7	0·8	4·5	9·7	0·3	—	0·5	0·2	tr.	1·4	3·5	17·6	tr.	0·4	—	1·0	2·8	11·5	0·3	8·5
		,,	(2) Dec.	0·9	0·3	30·4	4·2	0·7	0·7	5·3	11·0	0·3	—	—	tr.	—	—	5·4	20·8	—	0·6	—	0·7	5·5	13·2	—	5·7
Halibut	Fresh	Neutral lipid	(1) July	0·8	0·4	9·6	2·5	0·6	3·0	9·0	12·3	tr.	tr.	—	tr.	0·1	4·0	14·4 (−8H + −10H)		0·1	2·8	—	2·8 (−8H + −10H)		37·6	—	1·05
	,,	,, ,,	(2) Dec.	2·3	0·6	12·9	5·3	1·2	3·9	4·2	17·1	—	—	1·4	0·4	0·8	7·3	0·9	12·6	0·5	5·0	—	1·5	2·3	19·2	0·6	3·0
	,,	Phospholipid	(1) July	0·4	—	17·2	1·5	0·9	5·1	6·7	7·1	tr.	—	—	0·1	—	1·0	3·4	8·1	—	—	—	3·3	tr.	45·2	—	2·6
	,,	,,	(2) Dec.	0·8	0·2	19·0	2·1	1·6	2·7	7·7	9·9	0·7	—	0·4	0·7	tr.	2·8	2·7	10·3	—	—	—	—	1·5	36·9	—	2·5
	11·5 weeks, −14°	FFA	(1) July	1·2	0·2	16·5	7·1	1·1	1·6	3·0	19·4	2·0	—	1·5	0·2	—	7·2	4·0	14·9	0·3	3·7	—	tr.	tr.	16·1	—	5·5
Dogfish	Fresh	Neutral lipids	(1) Dec.	1·8	0·7	17·0	4·6	1·7	4·3	2·9	16·1	2·2	—	1·3	0·8	0·5	5·2	2·7	8·3	0·4	4·0	—	0·8	2·7	21·8	0·2	5·9
	,,	Phospholipids	(1) Dec.	0·4	0·6	19·6	2·8	2·0	2·9	8·8	10·7	0·7	—	—	0·6	1·0	3·1	7·0	5·1	—	—	—	0·5	2·3	32·0	—	2·2
	,,	,,	(2) July	0·4	—	17·6	1·8	0·6	3·6	8·7	10·0	tr.	—	—	0·2	tr.	2·9	4·7	9·3	—	tr.	—	—	3·5	36·7	—	2·0
	11·5 weeks, −14°	FFA	(2) July	1·0	0·3	16·2	3·2	2·5	0·9	2·7	19·8	1·8	—	1·2	0·5	—	6·7	4·3	16·1	—	4·6	—	—	—	18·2	—	6·0
Dogfish liver	Fresh	Neutral lipids	Dec.	3·0	0·6	15·0	4·9	1·0	2·9	3·4	19·0	1·4	—	1·4	0·6	0·3	9·5	1·1	5·4	0·5	14·0	—	1·0	2·1	12·2	0·7	4·4
Haddock[e]	11·5 weeks, −14°	FFA	July	0·6	0·4	32·0	1·8	0·7	0·8	2·8	8·3	0·7	0·4	0·3	0·1	—	1·1	3·2	16·9	0·2	tr.	—	—	2·2	27·5	—	11·4
		,,	July	1·0	0·4	33·0	2·4	0·5	2·6	3·1	9·5	1·3	1·1	0·7	0·3	—	1·0	2·4	16·9	0·1	0·2	—	—	2·8	20·7	—	10·6

[a] for Latin names for species see reference 6 [b] saturated fatty acid [c] 1 double bond [d] Palmitic/stearic acid ratio
[e] *Gadus aeglefinus* tr. = trace

C_{16} fatty acids, for example, have been found to be particularly effective in altering the biochemical properties of cytochrome *c*.[29]

Oxidised fatty acids are more effective in aggregating cod myosin than unoxidised acids,[30a] and also in insolubilising mackerel muscle proteins.[31] Desai & Tappel[32] have shown that 2·5 mol. of peroxy radical will cause the insolubilisation of a cytochrome-*c* molecule and therefore by analogy 10–15 mg. of fatty acid peroxide would be all that would be required to denature all the myosin (mol. wt. 500,000[33]) of 100 g. of cod muscle. This would be equivalent to a peroxide value of ~60 but, in denaturing protein, peroxide could break down and not be detected.

Table I shows that there were no highly significant trends either in the nature of the FFA produced during cold storage or in the composition of the phospholipids or neutral lipids from which the FFA derived, which could account for the different rates of protein denaturation in the cod, dogfish, halibut and sole, k $weeks^{-1}$ 0·13, 0·05, 0·03 and 0·014 respectively.[34] However, a closer examination of the data showed that the overall unsaturation of the FFA did decrease with increasing protein stability and a large contribution to this decrease was caused by the C_{22} hexaene content of the FFA, viz., 30·6, 18·2, 16·1 and 12·3% respectively. The latter addition of two samples of FFA from haddock stored for 11·5 weeks at $-14°$ contraindicated this relationship; 21–27% C_{22} hexaene for a rate of protein denaturation k $weeks^{-1}$ of 0·029.[34] (k is derived from the first-order curves fitted to the experimental determinations of protein solubility vs storage time at the same temperature.)

It should be emphasised that the lipid analyses were not done on the same samples of fish from which the protein data were obtained. The latter measurements may change considerably at different periods of the year, as also may the lipid composition of the neutral lipids and phospholipids. The remarkable similarity between the phospholipid analyses of our North Sea cod and that of a Nova Scotian sample[35] is encouraging. However, the analyses in the present paper for the phospholipids of halibut caught in July and December are not so close, and the analyses by Shuster *et al.*[23] from one albacore to another were markedly different. These workers noted some oxidation of lipids during the analytical treatment despite the use of hydroquinone during extraction procedures.

The only indication from this work is that the C_{22} hexaene in the FFA might be of more than average significance in protein denaturation and in any future studies the closest attention should be paid to the prevention of oxidation during the analytical procedures. Recovery of FFA has been shown previously[5] to accord with the amount of phospholipid hydrolysed in cold stored cod. Assuming a 75% hydrolysis of cod phospholipids after 26 weeks at $-14°$,[25] a summation of the amounts of individual fatty acids of the remaining phospholipids and of the FFA produced by phospholipid breakdown agreed with the fatty acid composition of the original fresh phospholipids. The present work, therefore, indicates that a preferential oxidation of polyunsaturated fatty acids had not occurred during cold storage of cod and in the subsequent analytical procedures. However, Castell[36] has found that susceptibility of cod muscle to metal-catalysed oxidation varies markedly at different times of the year; whether the observation also applies to extracted lipids is not known. The phospholipids isolated from cod muscle can certainly be highly susceptible to oxidation.[30b]

Acknowledgments

The authors would like to acknowledge the help which they obtained from Drs. M. Steinberg, F. King, M. Anderson and E. Gould of the Bureau of Commercial Fisheries, Gloucester, Mass., for making their data available prior to publication.

The work described in this paper was carried out as part of the programme of the Dept. of Scientific & Industrial Research. Thanks are due to Dr. D. P. Cuthbertson, C.B.E., for allowing part of this work to be carried out at the Rowett Research Institute.

Torry Research Station (D.S.I.R.)
Aberdeen
and
Rowett Research Institute
Bucksburn
Aberdeen

(Crown Copyright Reserved)

Receive 3 July, 1964

References

[1] 'Fish in Nutrition', 1962, p. 151 (Heen, E., & Kreuzer, R., ed.) [London : Fishing News (Books) Ltd.]
[2] Hanson, S. W. F., & Olley, J., in 'FAO Symposium on the Significance of Fundamental Research in the Utilisation of Fish', 1964 (in preparation)
[3] Anderson, M. L., Steinberg, M. A., & King, F. J., as reference 2
[4] Dyer, W. J., & Fraser, D. I., *J. Fish. Res. Bd Can.*, 1959, **16**, 43
[5] Olley, J., & Lovern, J. A., *J. Sci. Fd Agric.*, 1960, **11**, 644
[6] Olley, J., Pirie, R., & Watson, H. A., *J. Sci. Fd Agric.*, 1962, **13**, 501
[7] King, F. J., Anderson, M. L., & Steinberg, M. A., *J. Fd Sci.*, 1962, **27**, 363
[8] Anderson, M. L., & Steinberg, M. A., personal communication
[9] Anderson, M. L., & Steinberg, M. A., *J. Fd Sci.*, 1964, **29**, 327
[10] Lovern, J. A., as reference 1, p. 86
[11] McCarthy, R. D., & Duthie, A. H., *J. Lipid Res.*, 1962, **3**, 117
[12] Farquhar, J. W., Insull, W., jun., Rosen, P., Stoffel, W., & Ahrens, E. H., jun., *Nutr. Rev.*, 17, 1959, Suppl., p. 1
[13] Silk, M. H., & Hahn, H. A., *Biochem. J.*, 1954, **56**, 406
[14] Popják, G., & Tietz, A., *Biochem. J.*, 1954, **56**, 46
[15] Gray, G. M., & MacFarlane, M. G., *Biochem. J.*, 1961, **81**, 480
[16] Cardin, A., Bordeleau, M. A., & Laframboise, A., *J. Fish. Res. Bd Can.*, 1958, **15**, 555
[17] Richardson, T., Tappel, A. L., Smith, L. M., & Houle, C. R., *J. Lipid Res.*, 1962, **3**, 344
[18] Richardson, T., Tappel, A. L., & Gruger, E. H., *Arch. Biochem. Biophys.*, 1961, **94**, 1
[19] Lovern, J. A., *Biochem. J.*, 1937, **31**, 755
[20] Klenk, E., & Eberhagen, D., *Hoppe-Seyl. Z.*, 1962, **328**, 180
[21] Guha, K. D., Hilditch, T. P., & Lovern, J. A., *Biochem. J.*, 1930, **24**, 266
[22] Malins, D. C., Wekell, J. C., & Houle, C. R., *J. Lipid Res.* (in the press)
[23] Shuster, C. Y., Froinies, J. R., & Olcott, H. S., *J. Amer. Oil Chem. Soc.*, 1964, **41**, 36
[24] Bligh, E. G., *J. Fish. Res. Bd Can.*, 1961, **18**, 143
[25] Lovern, J. A., & Olley, J., *J. Fd Sci.*, 1962, **27**, 551
[26] Putnam, F. W., *Advanc. in Protein Chem.*, 1948, **4**, p. 80 (New York : Acad. Press Inc.)
[27] Boyer, P. D., Ballon, G. A., & Luck, J. M., *J. biol. Chem.*, 1947, **167**, 407
[28] Machebœuf, M. A., & Tayeau, F., *Bull. Soc. Chim. biol.*, 1941, **23**, 49
[29] Hardesty, B. A., & Mitchell, H. K., *Arch. Biochem. Biophys.*, 1963, **100**, 1
[30] (*a*) Torry Research Sta., Annu. Rep., 1962, p. 31 (Edinburgh : H.M.S.O.) ; (*b*) *ibid.*, p. 34
[31] Ota, F., & Nishimoto, J., *Bull. Inst. int. Froid*, 1963, Annexe IV–13
[32] Desai, I. D., & Tappel, A. L., *J. Lipid Res.*, 1963, **4**, 204
[33] Connell, J. J., & Mackie, I. M., *Nature, Lond.*, 1964, **201**, 78
[34] Love, R. M., & Olley, J., in 'FAO Symposium on the Significance of Fundamental Research in the Utilisation of Fish', 1964 (in preparation)
[35] Ackman, R. G., & Burgher, R. D., *J. Fish. Res. Bd Can.*, 1964, **21**, 367
[36] Castell, C. H., as reference 34

DISTILLATION OF PYRETHRUM EXTRACT IN A WIPED-WALL, FALLING-FILM, SHORT-PATH STILL: SEPARATION OF 'PYRETHRIN I' AND 'PYRETHRIN II' AND DETERMINATION OF THEIR RELATIVE BIOLOGICAL ACTIVITIES

By A. A. GOLDBERG, S. HEAD and (Mrs.) P. JOHNSON

Pyrethrum oleoresin has been distilled in an industrial 12-in. wiped-wall, falling-film, short-path still in order to produce a decolorised dewaxed pyrethrum extract. The distilled material was free from isopyrethrins and a series of bio-assays showed the biological activities of the distilled and undistilled extracts to be identical.

Substantially pure 'Pyrethrin I' and 'Pyrethrin II' have been obtained by fractionation of pyrethrum extract in a similar 2-in. still. It was found that 'Pyrethrin II' has 3·2–4·0 times the knockdown activity of 'Pyrethrin I' against *M. domestica*; when synergised 5 : 1 with piperonyl butoxide, however, 'Pyrethrin II' has only 1·9 times the knockdown activity of similarly synergised 'Pyrethrin I' against *M. domestica*. 'Pyrethrin II' has 0·95, 0·55 and 0·74 times the lethal activity of 'Pyrethrin I' against *M. domestica, T. castaneum* and *C. oryzae* respectively.

Introduction

The problem encountered in the production of a decolorised pyrethrum extract by distillation of pyrethrum oleoresin is the avoidance of conditions under which isomerisation of the pyrethrins to isopyrethrins occurs with consequent loss in biological activity. The thermal isomerisation of the pyrethrins to isopyrethrins is a first-order reaction[1] with energy and entropy of activation respectively 24,610 cal. mol.$^{-1}$ and $-21{\cdot}8$ cal. deg.$^{-1}$ and with a rate constant $k = 4{\cdot}81 \times 10^{-4}$ at 195°. It is evident from the values of these parameters that distillation can be effected without measurable isomerisation taking place provided the heating period at elevated temperatures is not more than a few seconds' duration. With the recent availability of wiped-wall, short-path, falling-film, high-vacuum stills this requirement can be fully satisfied.

Pyrethrum oleoresin has previously been distilled[2-5] in short-path falling-film laboratory stills in order to obtain a decolorised non-staining extract. Goldberg & Smith[4] found by measurement of the ratio of the optical densities at 2700 and 2300 Å that distillation could be effected without isomerisation. Elliott *et al.*,[5] who used a 2-in. wiped-wall laboratory still, recorded the absence of isopyrethrins in the distillate and established that the LD_{50} values of the undistilled and distilled extracts to mustard beetles (*Phaedon cochleariae*) were identical. Pyrethrum oleoresin has been co-distilled[6] with an excess of piperonyl butoxide on an industrial scale for several years. The present communication records observations made during the period 1960–62 when 200,000 lb. of pyrethrum 25% oleoresin was distilled in a 12-in. wiped-wall, short-path still; the distillate was free from isopyrethrins and a long series of bio-assays showed no statistically significant difference between the biological activities of the distilled and undistilled material.

Distillation in a 12-in. wiped-wall, short-path, falling-film industrial still

The still, manufactured by Edwards High Vacuum Ltd., has been described[7] with full engineering specifications and only brief details are necessary here. The distilland (feed), contained in a 60-gal. vessel A (Fig. 1) mounted on a weighing machine, passes via the variable-flow measuring pump B through the first (DG_1) and second (DG_2) degassers where all low-boiling material is stripped off; it then passes into the 12-in. evaporator E where the actual distillation takes place. The degassers, DG_1 and DG_2, are each 15 cm. i.d. and 30 cm. long and are fitted with three slotted wiper blades equally spaced at 120° in a loose-fitting cage rotating at 400 r.p.m. and kept in contact with the cylinder walls by centrifugal force. The evaporator E is 30 cm. in diameter and 50 cm. long, fitted with four slotted wiper blades equally spaced at 90° and rotating at 120 r.p.m. The rotating wiper blades are designed to accelerate the film downwards in order to decrease the concentration gradient and decrease the time of exposure of the film to the heated cylinder wall. In all three vessels DG_1, DG_2 and E the feed descends as a thin film on the cylinder wall under the influence of the rotating slotted wiper blades: distillation takes place from the hot wall through the wiper blades on to a central axial condensing finger.

Theoretical

Let ABCD (Fig. 2) be a vertical section of the cylindrical evaporator. Distilland enters at B and travels down the heated wall of the still as a continuous thin film. Consider a very

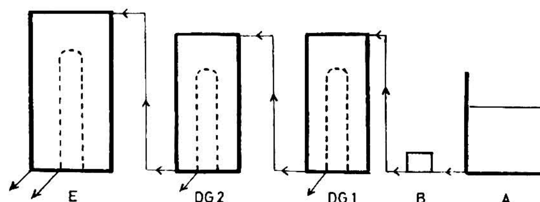

FIG. 1.—*Flow sheet of distillation of pyrethrum concentrate*

A feed vessel
B measuring pump
DG1, DG2 degassers
E evaporator

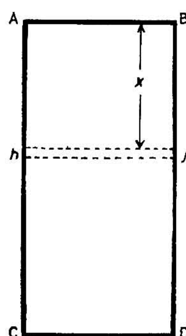

FIG. 2

narrow annular segment h_j of the film of volume v at distance x from the top. Then $v = 2\pi r\alpha\beta$ where r is the radius of the cylinder, and α and β respectively the width and thickness of the annular film segment. Let this annular segment of film with pyrethrin concentration c move down the still wall a distance dx. For a given rate of heat transfer across the still wall the amount of pyrethrins which distil $\mathrm{d}P(= -v\,\mathrm{d}c)$ will be proportional to the total amount of pyrethrins vc in the segment and the distance travelled dx.

That is, $$\mathrm{d}P = \mathrm{k}\,vc.\mathrm{d}x = -v\,\mathrm{d}c$$

$$\therefore\ \mathrm{d}c/c = -\mathrm{k}\,\mathrm{d}x$$

Integration gives $$\ln c = -\mathrm{k}x + \mathrm{K}$$

When $x = 0$, $c = c_0$ the concentration of pyrethrins in the distilland entering the still

$$\therefore\ \ln c/c_0 = \mathrm{k}x$$

$$c = c_0\,\mathrm{e}^{-\mathrm{k}x} \qquad (1)$$

The amount of pyrethrins P which has distilled from this annular segment of film during its travel from AB to h_j is given by

$$P = v_0c_0 - vc$$

$$= c_0(v_0 - v\mathrm{e}^{-\mathrm{k}x}) \qquad (2)$$

where v_0 is the volume of the annular segment when $x = 0$.

It is necessary to determine v in terms of x. As the annular segment at h_j travels down the wall a distance of dx, the decrease in volume $-\mathrm{d}v$ will be proportional to v and to dx.

$$-dv = \mathrm{k}_1.v.\mathrm{d}x$$

$$\therefore\ \mathrm{d}v/v = -\mathrm{k}_1.\mathrm{d}x$$

Hence $$\ln v = -\mathrm{k}_1x + \mathrm{K}$$

When $$x = 0,\ v = v_0$$

$$\therefore\ v = v_0\,\mathrm{e}^{-\mathrm{k}_1x} \qquad (3)$$

Substitution of this value of v in Equation (2) gives

$$P = c_0(v_0 - v_0\mathrm{e}^{-\mathrm{k}x}.\mathrm{e}^{-\mathrm{k}_1x})$$

$$= v_0c_0(1 - \mathrm{e}^{-\gamma x}) \qquad (4)$$

where γ is a constant.

The total distillate for the whole run ΣP is the sum of the pyrethrins which distil from all such segments.

$$\Sigma P = \Sigma\, v_0\{c_0(1 - \mathrm{e}^{-\gamma x})\}$$

$$= Vc_0(1 - \mathrm{e}^{-\gamma x}) \qquad (5)$$

where V is the *total* volume of, and Vc_0 the total amount of pyrethrins in, the distilland.

Equation (5) governs the distillation: the constant γ, which depends upon the rate of flow of the distilland and the rate of heat transfer across the still wall, can be determined from

one distillation under standardised conditions. For example, with the standard conditions used in which 80% of the input pyrethrins distil over during the first pass (see below)

$$\Sigma (P)/Vc_0 = 0{\cdot}8 = (1 - e^{-50\gamma})$$

which gives $\gamma = 0{\cdot}032$

The mean thickness of the film on the evaporator wall is of the order of 0·1 mm. The volume of film on the hot wall at any one time will therefore be (95 × 50 × 0·01) c.c. = 48 c.c. With an input rate to the still of 30 l./h. the time of contact of the film on the hot wall will be approximately 6 sec. The mean time of contact of the *pyrethrin molecules* with the hot wall will be less than this because the rate of distillation of the pyrethrins is higher at the top than at the lower end of the evaporator. From Equation (4) it follows that, if x_1 is the distance down the still wall in which one half of the total distilled pyrethrins come over,

$$\Sigma P/v_0c_0 = 0{\cdot}4 = (1 - e^{-\gamma x_1})$$

which gives $x_1 = 15{\cdot}8$ cm. Accordingly the mean time of contact of the pyrethrin molecules as they pass through the still under these standard conditions will be

$$2 \times 15{\cdot}8 \times 6/50 = \sim 4 \text{ sec.}$$

The isomerisation of the pyrethrins to isopyrethrins is a first-order reaction with a rate constant $k = 4{\cdot}81 \times 10^{-4}$ at 195°. From the Arrhenius equation it follows that at 225°, the normal temperature of the evaporator, $k = 2{\cdot}36 \times 10^{-3}$; accordingly, for a heating time of 4 sec. at 225°, the amount of pyrethrins which will be isomerised is ~1·0%. From previous work[1] on the comparative activities of the pyrethrins and isopyrethrins, the loss in biological activity will be of the order of 0·5% ; this is far too small to detect even by the most ambitious method of bio-assay.

Experimental

After a considerable number of experimental runs to determine optimum conditions for maintaining high distillation yields without damaging the pyrethrins by thermal isomerisation, the following conditions were established and used for the distillation of ~200,000 lb. of pyrethrum extract.

Each distillation batch was a unit of ~100 lb. of pyrethrins in the form of 300–310 lb. of 32–33% oleoresin concentrate, 50 lb. of Light Liquid Paraffin B.P. and 25 lb. of Liquid Paraffin B.P. These were weighed into the reservoir A, the mixture stirred until homogeneous, and sampled. Distillation was carried out with the following settings : (i) Flow rate of distilland : 28–32 l./h. ; (ii) first degasser : 150°/3–5 mm. ; (iii) second degasser : 125–150°/10–15 μ McLeod ; (iv) evaporator : 225–230°/2–4 μ McLeod (12–14 μ Pirani). The temperatures recorded in the two degassers were those of the distilland and in the evaporator that of the cylinder wall. With the high rate of passage of the film down the cylinder wall and the localised effect of the latent heat of vaporisation it is doubtful whether the temperature of the pyrethrin molecules in the evaporator reached 200°.

The distillate (180–200 lb. containing 80–82% of the input pyrethrins) was collected in a tared 40-gal. steel drum and the undistilled residue (170–190 lb.) in a receiver where it was diluted with 30 lb. of Light Liquid Paraffin B.P. and 15 lb. of Liquid Paraffin B.P. and passed through the still again. The distillate from the second pass (70–90 lb. containing 11–12% of the initial input pyrethrins) was collected in the drum containing the first pass distillate, the mixture diluted with sufficient kerosene to give a clear pale yellow solution containing 25–26% of pyrethrins and stirred for 2 h. before being sampled. The drum was then sealed and stored in the cold room at −5° for the dewaxing process. The second pass residue (120–130 lb.) contained ~6% of the input pyrethrins which were damaged as shown by the d_{2700}/d_{2300} ratio ;* this material, when cooled, set to a black bituminous mass and was rejected as useless. Each drum of distilled material was analysed spectroscopically and the d_{2700}/d_{2300} ratio determined : this was always between the limits 0·075–0·085 showing the absence of isopyrethrins and the retention[1] of full activity.

Under the above distillation conditions Light Liquid Paraffin B.P. distils completely and

* ratio of optical densities at 2700 and 2300 Å

facilitates the distillation of the pyrethrins by acting as co-distillant : Liquid Paraffin B.P. does not distil at 230°/5 μ and serves the purpose of keeping the film of distilland on the evaporator wall more fluid and lubricating the line of contact of the wiper blades with the wall. Distillation of a mixture of these two paraffins is useful for cleaning the interior of the still and flushing through residual pyrethrins held in the pipe system.

A considerable number of carefully controlled determinations of yield were made. In one of these the still was washed by distilling 20 gal. of the mixed liquid paraffins and then 6 × 100 lb. pyrethrin unit batches were distilled ; the still was then washed through by distilling a mixture of the two paraffins until the distillate was free from pyrethrins. The distillates were weighed, homogenised and samples taken ; these, together with the samples of the feed were analysed by the P.B.K.,[8] A.O.A.C.,[9] D.N.P.[10] and spectroscopic methods by two groups of analysts. The mean recorded yield by the four methods was 92·8, 93·1, 95·3 and 93·0% respectively.

Tables I and II exemplify the effects of varying the evaporator temperature, the relative amount of the co-distilling Light Liquid Paraffin B.P. and the rate of the feed to the still. With the smaller amount of co-distillant, the ratio of the pyrethrins in the distillate to pyrethrins in the residue increased as the evaporator temperature increased from 210 to 260°. With the larger amount of co-distillant there was virtually no increase ; just over 80% of the input pyrethrins distilled during the first pass over the range 210–260° even although the rate of feed was increased from 18 to 32 l./h. It is also of interest that the d_{2700}/d_{2300} ratio of the material distilling through the evaporator at 260° was 0·075 which shows absence of isomerisation to isopyrethrins and therefore retention of full biological activity (see Table IV, footnote).

In one experiment the second degasser temperature was increased stepwise and, after the still had reached equilibrium under the new setting, samples taken for spectroscopic examination. When the second degasser temperature reached 180° the d_{2700}/d_{2300} ratio rose to 0·11 showing that isomerisation had commenced. Accordingly all bulk distillations were carried out with the second degasser set at not more than 150°.

Table I

Distillation of pyrethrum concentrate in 12-in. still

All variables kept constant except evaporator temperature which was raised from 210° to 260°/4 μ (McLeod) 1st degasser : 150–160°/4 mm. ; 2nd degasser : 80–85°/20μ Feed rate : 18 l./h.

Feed : 296·9 lb. of oleoresin 32·28% Py. ; 15·8 lb. Light Liquid Paraffin B.P. ; 16·3 lb. Liquid Paraffin B.P

(1) Time, p.m.	(2) Evaporator temp., °C	(3) Distillate rate, l./h.	(4) Residue rate, l./h.	(5) Distillate analysis (PBK)			(6) Residue analysis (PBK)			(7) Ratio of pyrethrins distilling to pyrethrins not distilling	(8) d_{2700}/d_{2300} ratio of distillate
				Py. I %	Py. II %	Total %	Py. I %	Py. II %	Total %		
3·10	210°	8·5	9·5	31·38	19·90	51·28	6·06	7·01	13·07	3·54	0·065
4·45	220°	8·5	9·5	30·55	19·92	50·47	6·22	6·61	12·83	3·55	0·064
5·45	230°	9·5	9·0	29·96	19·76	49·72	5·86	6·43	12·29	4·28	0·070
6·45	240°	9·5	8·5	29·79	19·71	49·50	5·63	5·93	11·56	4·76	0·070
8·20	250°	9·5	8·5	28·97	19·72	48·69	5·52	6·05	11·57	4·72	0·072
9·25	260°	9·8	8·2	27·99	18·42	46·41	5·32	5·70	11·02	5·02	0·075

Total distillate : 153·1 lb. (Py. I 28·80% ; Py. II 19·24% ; Total Py. 48·04%) = 73·6 lb. pyrethrins = 77·2% of feed

Total residue : 106·6 lb. (Py. I 5·52% ; Py. II 6·20% ; Total Py. 11·72%) = 18·8 lb. pyrethrins = 19·7% of feed

The residue was diluted with 30 lb. of Light Liquid Paraffin and 15 lb. of Liquid Paraffin and passed through the still with the evaporator at 250°/5 μ at a rate of 25 l./h.

Second pass distillate : 81·25 lb. (Py. I 9·65% ; Py. II 8·73% ; Total Py. 18·38%) = 14·97 lb. pyrethrins = 15·7% of initial Feed (d_{2700}/d_{2300} ratio 0·09)

Second pass residue : 120·9 lb. (Py. I 2·02% ; Py. II 2·54% ; Total Py. 4·56%) = 5·52 lb. pyrethrins = 5·8% of initial feed (d_{2700}/d_{2300} ratio 0·34)

Total yield of distillate : 92·9% of feed. The combined distillates had d_{2700}/d_{2300} ratio 0·078

(in this and later Tables Py. = pyrethrins)

Table II

Distillation of pyrethrum concentrate in 12-in. still (second series)

All variables kept constant except evaporator temperature which was raised from 210°–260°/5 μ (McLeod) 1st degasser: 145–155°/3 mm.; 2nd degasser: 110–125°/40 μ. Feed rate: 30–32 l./h.

Feed: 297·6 lb. of oleoresin (Py. I 17·96%; Py. II 14·14%; Total Py. 32·10%) plus 49·9 lb. of Light Liquid Paraffin B.P. plus 16·8 lb. of Liquid Paraffin B.P.

(1) Time, p.m.	(2) Evaporator temp., °C	(3) Distillate rate, l./h.	(4) Residue rate, l./h.	(5) Distillate analysis (PBK)			(6) Residue analysis (PBK)			(7) Ratio of pyrethrins distilling to pyrethrins not distilling	(8) d_{2700}/d_{2300} ratio of distillate
				Py. I %	Py. II %	Total %	Py. I %	Py. II %	Total %		
7·30	210°	15·95	16·7	28·05	18·27	46·32	4·86	6·02	10·88	4·09	0·066
8·30	220°	16·2	16·4	27·82	18·17	45·99	4·94	5·97	10·91	4·19	0·066
9·15	230°	16·3	15·6	26·07	17·85	43·87	5·32	5·89	11·21	4·09	0·074
10·15	240°	16·7	16·0	26·21	18·04	44·25	5·23	5·80	11·03	4·17	0·070
11·10	250°	16·0	14·7	25·29	17·68	42·97	5·30	6·11	11·41	4·10	0·078
12·10	260°	17·1	15·0	25·10	17·26	42·36*	5·48	5·96	11·44	4·22	0·081

1st pass total distillate 177·1 lb. (Py. I 26·52%; Py. II 17·80%; Total Py. 44·32%) = 82% of pyrethrins in initial feed

Dewaxing of the distillate

The distilled material after dilution with kerosene to 25–26% pyrethrins was a clear pale yellow liquid at 26° (the ambient temperature at Nakuru) which became cloudy at 10–15° and opaque with precipitated wax at 0°. After storage of the drums in the cold room for several days at −5° the contents were stirred and pumped through a Sharples centrifuge (15,000 r.p.m.) at a rate of ~30 gal./h.; the clear liquid emerging from the centrifuge was transferred to a 500-gal. weighing tank. For each 100 lb. of this solution 0·5 lb. of 2,6-di-t-butyl-*p*-cresol (Topanol OC antioxidant) together with 5 lb. of perfumery grade terpineol or absolute isopropyl alcohol was added and the solution then diluted to 20·0% pyrethrins with kerosene. The semi-solid wax from the centrifuge (~20 lb. with 25–30% pyrethrin content from each 40-gal. drum) was extracted in bulk several times with hot kerosene, the extract clarified by centrifuging and used for diluting material from the still.

Characteristics of product

The final product had the following properties:

(i) colour: pale yellow liquid of approximately the same colour value as brandy or whisky

(ii) density: 0·87 g./c.c. at 22°;

(iii) viscosity: 8·2 centistokes at 22° (kinetic);

(iv) d_{2700}/d_{2300} ratio: 0·080–0·085;

(v) wax content: ~40 lb. of oleowax per 100 lb. pyrethrins as against 180 lb. of oleowax per 100 lb. of pyrethrins in 25% pyrethrum oleoresin extract;

(vi) stability: after being kept for 2 years in amber bottles or metal canisters in an unheated store in London, the extract remained crystal clear and free from sediment: in clear glass bottles exposed to sunlight the liquid rapidly became cloudy;

(vii) solubility: a 2% pyrethrins solution in 95% kerosene and 5% methylene chloride or isopropyl alcohol was starbright at 0°; a solution comprising 20% pyrethrins extract (1·5%), piperonyl butoxide (1·5%), methylene chloride (2·0%), kerosene (15%), dichlorofluoromethane, (40%) and trichlorofluoromethane (40%) was crystal clear at −30°. The chysanthemic acid obtained by hydrolysis of the extract had b.p. 114–116°/1 mm. and $[\alpha]_D^{20}$ + 14·31° (ethanol).

The yield was ~86% of the input pyrethrins to the still plus ~1% of undamaged pyrethrins in the centrifuge wax residues. It is worthy of record that a preferable (although costlier) method of dewaxing, which could be applied either before or after the distillation, comprised the addition of 95% methyl or ethyl alcohol until the solution contained ~5% of pyrethrins, freezing to -5°, centrifuging off the precipitated wax and then distilling off the solvent.

Biological activity of the distilled extract

The biological activity of the distilled extract was compared with that of undistilled oleoresin by (i) the Kearns & March[11] method for knockdown and kill of flies (Tables III and IV), (ii) topical application of a measured drop for the kill of flies (Table V) and (iii) the dusted wheat method[12] for the kill of grain weevils (Table VI). The results show conclusively that the activities of the distilled and undistilled extracts are identical. This confirms previous findings[1] that the biological activity of a pyrethrum extract which has been subjected to heat treatment is a function of the ratio of the optical density of the extract at 2700 Å to that at 2300 Å and that provided this ratio does not exceed a value of 0·095 no damage has been effected by the heat treatment.

Table III

Knockdown of M. domestica *by distilled and undistilled pyrethrum extract in the Kearns & March chamber*

5-Day-old flies: 80–100 flies per test: 0·2 c.c. of the solution of the toxicant in kerosene sprayed from each nozzle at 12·5 p.s.i. during 5 sec.: 8 or 9 replicates for each test

% knockdown (10 min.)					
0·02% pyrethrins (unsynergised)			0·01% pyrethrins + 0·05% piperonyl butoxide		
Distilled	Undistilled	Difference	Distilled	Undistilled	Difference
57	65	−8	84	81	+3
76	76	0	74	65	+9
43	56	−13	70	65	+5
44	33	+11	45	49	−4
27	26	+1	42	45	−3
46	57	−11	60	48	+12
28	25	+3	78	69	+9
76	64	+12	41	52	−11
70	79	−9	—	—	—
M, 52	M, 54	MD, −2	M, 62	M, 59	MD, +3

For the mean difference (MD) [0·02% pyrethrins]
Standard deviation: 9·3
Standard error: 3·1
95% confidence limits: +5·2 to −9·2

For the mean difference (MD) [0·01% pyrethrins + 0·05% piperonyl butoxide]
Standard deviation: 7·9
Standard error: 2·8
95% confidence limits: +9·8 to −3·6

It is of interest to record that the Wisconsin Alumni Corporation examined samples of the above distilled and undistilled pyrethrum extract and reported the following:

LD_{50}: topical application to female flies: distilled 0·78 μg./fly: undistilled 0·78 μg./fly. Two official Peet–Grady tests using 0·02% pyrethrins in each case (500 flies per test) gave the figures shown in Table VII.

Separation of 'Pyrethrin I' and 'Pyrethrin II' by fractional distillation in a 2-in. wiped-wall, falling-film still

'Pyrethrin I' and 'Pyrethrin II' have batch boiling points of the order of 170°/0·1 mm. and 200°/0·1 mm. respectively and it is evident that, provided constant-boiling mixtures are not formed, considerable separation should be effected by fractional distillation. The presence of a co-distillant such as Light Liquid Paraffin B.P. effectively lowers the boiling points of both components.

The still, manufactured by Edwards High Vacuum Ltd., has been previously[5] described. The evaporator consisted of a glass cylinder, 5 cm. dia. and 20 cm. long, the inside of which was wiped by three slotted PTFE blades rotating at 120 r.p.m.; the outside was heated by an electrothermal jacket which could be set at any prearranged temperature. The feed, previously degassed on a water pump, was stripped of low-boiling material by passage through the still at 80–100°/1–2 mm. and the undistilled residue then repeatedly passed through at ascending temperatures under high vacuum. Tables VIII–X, which are self-explanatory, show the degree of separation possible. With reference to their total pyrethrin content the third (D3) and seventh (D7) fractions of Table VIII consisted of Py. I 93·5%/Py. II 6·5% and Py. II 89·4%/Py. I 10·6% respectively; these were considered to be substantially pure 'Pyre-

Table IV

Knockdown and kill of M. domestica *by distilled and undistilled pyrethrum extract in the Kearns & March chamber*

5-Day-old flies : 80–100 flies per test : 0·2 c.c. of the solution of the toxicant in kerosene sprayed from each nozzle at 12·5 p.s.i. during 5 min. : 8 replicates each test. Flies kept in jars supplied with 5% sugar solution and mortality count determined after 24 h.

% knockdown in 5 min.

0·01% pyrethrins + 0·05% piperonyl butoxide			0·02% pyrethrins + 0·10% piperonyl butoxide		
Distilled	Undistilled	Difference	Distilled*	Undistilled	Difference
32	25	+7	56	50	+6
17	21	−4	58	86	−28
32	23	+9	56	54	+2
14	18	−4	70	89	−19
16	18	−2	62	64	−2
22	22	0	88	74	+14
39	30	+9	82	65	+17
16	10	+6	66	70	−4
M, 24	M, 21	MD, +3	S, 67	S, 69	MD, −2

For the mean difference (MD)
Standard deviation : 6·3
Standard error : 2·2
95% confidence limits : +8 to −2

For the mean difference (MD)
Standard deviation : 15·4
Standard error : 5·4
95% confidence limits : +11 to −15

% Kill (Males)

Distilled	Undistilled	Difference	Distilled	Undistilled	Difference
27	12	+15	65	57	+8
15	35	−20	71	91	−20
16	5	+11	70	94	−24
28	40	−12	90	88	+2
27	14	+13	86	77	+9
27	18	+9	81	84	−3
50	26	+24	76	71	+5
26	18	+8	80	99	−19
M, 27	M, 21	MD, +6	M, 77	M, 82	MD, −5

For the mean difference (MD)
Standard deviation : 15·9
Standard error : 5·6
95% confidence limits : +19 to −7

For the mean difference (MD)
Standard deviation : 13·6
Standard error : 4·8
95% confidence limits : +6 to −16

* Material distilled at 260°/5 μ (see Table II)

thrin I ' and ' Pyrethrin II ' and were used for the bio-assays. Solutions were prepared in a solvent consisting of kerosene (70%), ethyl methyl ketone (20%) and isopropyl alcohol (10%) and these examined in the Kearns & March Chamber for the knockdown of flies (Figs. 3 and 4), by topical application of a measured drop for the kill of flies (Table XI) and grain beetles (Table XII) and by the dusted wheat method for the kill of grain weevils (Table XIII).

Table V

Toxicity of distilled and undistilled pyrethrum extract to M. domestica *by topical application*

5-Day-old female flies were lightly anaesthetised with carbon dioxide and 2 × 0·1 μl. of the stated concentration of pyrethrins in kerosene applied to the ventral surface. The flies were then stored in jars supplied with 5% sugar solution and the mortality determined after 24 h. : 30 flies per dose ; 3 dose levels ; 5 replicates

Pyrethrins concn., %	Mean kill, % Distilled	Undistilled
0·8	97	95
0·6	71	76
0·4	49	50
LD_{50}, μg./fly (from graph)	0·80	0·80

Table VI

Toxicity of distilled and undistilled pyrethrum extract to C. oryzae *by dusted wheat method*

Dusts containing 0·5% pyrethrins on B.P. talc were prepared and an aliquot mixed with 50 g. of wheat. 50 weevils (7 days old) were added and mortality count made after 6 days ; 3 dose levels ; 5 replicates each dose

Pyrethrins (concn.) p.p.m.	Mean kill, % Distilled	Undistilled	Nitromethane decolorised extract
6	49	45	48
8	63	61	67
10	78	75	79
LD_{50}, p.p.m. (from graph)	6·64	6·74	6·40

Table VII

Extract	% knockdown		
	3 min.	5 min.	10 min.
Distilled	32	45	63
	43	47	59
	M, 38	46	61
Undistilled	44	51	65
	32	50	63
	M, 38	50	64

Discussion

The literature relating to the comparative biological activities of ' Pyrethrin I ' and ' Pyrethrin II ' is briefly surveyed in Table XIV. Although the relative activities would be expected to vary with the insect species, it is noticeable that there is considerable lack of consistency in the figures reported for the same insect. This is probably due to the employment of different carrier solvents, different techniques of application of the toxicant and, more significantly, to the use of ' Pyrethrin I ' and ' Pyrethrin II ', reconstituted from pyrethrolone and chryanthemic or pyrethric acid, and *batch distilled.* During this procedure there is high probability, particularly with the higher-boiling ' Pyrethrin II ', that partial isomerisation to isopyrethrins had taken place with lowering of activity.

The results of the present work (Figs. 3 and 4) show the knockdown activity of ' Pyrethrin II ' to be 4·0 and 3·2 times as high as that of ' Pyrethrin I ' at the KD_{50} (5 min.) and KD_{50} (10 min.) levels respectively. This ratio is in good agreement with the value of 3·5 recorded by Sullivan *et al.*[17] who observed that ' Pyrethrin II ' produces a faster and higher percentage knockdown but lower percentage of death than ' Pyrethrin I ' against both flies and cockroaches. Sawicki & Thain[26] reported a value of 3·1 for the ratio of the knockdown activities of pure Pyrethrin II and Pyrethrin I as determined by the method[27] of topical application of a

Table VIII

Separation of ' Pyrethrin I ' and ' Pyrethrin II ' by fractional distillation in 2-in. wiped-wall still

Initial feed : 450 g. of pyrethrum extract which had been bulk distilled in the 12-in. still. This was passed through the 2-in. still seven times under the conditions stated

Run. No.	1	2	3	4	5	6	7
Temperature, °c	100	110	120	130	140	150	160
Pressure, μ (Pirani)	30	13	8	8	8	9	9
Pressure, μ (McLeod)	20	10	2	1	1	1	1
Weight of feed to run, g.	450	380	320	277	230	175	122
Weight of distillate, g.	66	58	40	46	54	47	48
Weight of residue, g.	383	322	280	231	176	125	72*
Time for run, min.	60	60	52	40	45	34	32
Feed rate, g./min.	7·5	6·4	6·2	6·9	5·1	5·1	4·0
% Distilled on individual feed	14·5	15·2	12·5	16·6	23·6	26·8	39·2
% Distilled on initial feed (to Run 1)	14·5	12·9	8·8	10·2	11·9	10·4	10·6

* Final residue too viscous to distil

Analyses (PBK)

Material	Py. I, %	Py. II, %	Total Py., %	Py. I, g.	Py. II, g.	Py. I/Py. II ratio	d_{2700}/d_{2300} ratio
Initial feed	18·21	14·50	32·71	82·2	65·6	1·26	0·065
**D1	2·67	0·81	3·48	1·76	0·53	3·30	0·051
D2	16·06	1·27	17·33	9·34	0·74	12·67	0·048
D3	30·92	2·15	33·07	12·37	0·86	14·39	0·044
D4	40·42	4·92	45·34	18·60	2·26	8·21	0·048
D5	35·19	10·86	46·05	18·93	5·85	3·24	0·052
D6	19·98	26·46	46·44	9·44	12·46	0·76	0·066
D7	5·75	46·35	52·10	2·75	22·22	0·12	0·088

** D1 and R1 etc. mean distillate and residue of Run 1 respectively, etc., in this and following Tables

Table IX

Separation of ' Pyrethrin I ' and ' Pyrethrin II ' by fractional distillation in 2-in. wiped-wall still with co-distilland

Initial Feed : Equal volumes of 25% oleoresin extract, Light Liquid Paraffin B.P. and Liquid Paraffin B.P.

Run No.	1	2	3	4	5	6
Temperature, °C	100	120	127	135	145	155
Pressure, μ (Pirani)	80	25	11	10	11	10
Pressure, μ (McLeod)	2	1	1	1	1	1
Weight of feed to run, g.	380	314	228	153	95	50
Weight of distillate, g.	60	81	71	57	42	10
Weight of residue, g.	318	230	155	96	53	40
Time of run, min.	60	30	25	20	25	15
Feed rate, g./min.	6·5	10·5	9·5	7·6	3·8	3·4
% Distilled on individual feed	15·5	25·8	31·2	37·2	44·5	20·0
% Distilled on initial feed to run 1	15·4	20·7	18·4	14·6	10·8	2·6

Analyses (PBK)

Material	Py. I, %	Py. II, %	Total Py., %	Py. I content, g.	Py. II content, g.	Py. I/Py. II ratio	d_{2700}/d_{2300} ratio
Feed	3·71	2·80	6·51	14·10	10·60	1·32	0·082
D1	0·32	0·06	0·38	0·19	0·04	5·3	0·140
D2	2·05	0·21	2·26	1·66	0·17	9·75	0·053
D3	5·63	0·80	6·43	4·00	0·57	7·04	0·044
D4	8·98	2·75	11·73	5·13	1·56	3·26	0·042
D5	6·64	10·92	17·54	2·80	4·60	0·67	0·061
D6	2·64	18·80	21·44	0·26	1·89	0·14	0·088
R6	0	1·90	1·90	0	0·76	0	0·33

D1, D2, D3, D4 combined contain 78% of initial feed Py. I and 22·4% of initial feed Py. II; Py. I/Py. II ratio 4·7

D5 and D6 combined contain 22% of initial feed Py. I and 63% of initial feed Py. II ; Py. I/Py. II ratio 0·5

Total distilled pyrethrins = 96% of input pyrethrins

Table X

Separation of ' Pyrethrin I ' and ' Pyrethrin II ' by fractional distillation in 2-in. wiped-wall still

Initial feed : Equal volumes of 25% oleoresin extract, Light Liquid Paraffin B.P. and Liquid Paraffin B.P.

Run No.	1	2
Temperature, °C	90°	180°
Pressure, μ (Pirani)	200	20–25
Pressure, μ (McLeod)	40	1
Weight of feed to run, g.	380	250
Weight of distillate, g.	126	179
Weight of residue, g.	251	92
Time of run, min.	70	70
Rate of feed, g./min.	5·5	3·6

Analyses (PBK)

Material	Py. I, %	Py. II, %	Total Py., %	Py. I, g.	Py. II, g.	Total Py., g.	Py. I/Py. II ratio
Initial feed	5·05	3·91	8·96	19·2	14·9	34·1	1·29
D1	9·76	4·10	13·86	12·4	5·2	17·6	2·39
D2	3·30	4·73	8·03	5·97	8·47	14·44	0·70

% Input pyrethrins in D1 and D2 = 51·5% and 42·4% respectively

measured drop of toxicant to immobilised flies and classifying them as ' non-affected ' or ' affected ' according to whether the degree of paralysis was such that the insects were, or were not, able to fly/crawl after a given time interval. The agreement between the ratio obtained by this technique and that obtained in the Kearns & March chamber (Figs. 3 and 4) is of interest inasmuch as in the latter method the amount of toxicant picked up by a fly in the atomised mist must be a function of the primary activation threshold and the volume within which the insect is constrained for flight. The lower the activation threshold and the greater the permitted path of flight, the greater is the amount of toxicant absorbed upon the cuticle.

When synergised with a 5 : 1 ratio of piperonyl butoxide, however, the knockdown activity of ' Pyrethrin II ' is only 1·9 times that of similarly synergised ' Pyrethrin I ' at the KD_{50} (2·5 min.) and KD_{50} (5 min.) levels. It is apparent that for the rapid knockdown response

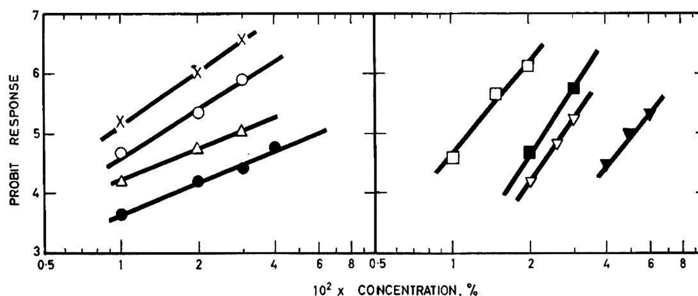

FIG. 3 (left).—*Knockdown of* M. domestica *by 'Pyrethrin I' and 'Pyrethrin II' in Kearns & March chamber*

80–100 Flies per test: 0·2 c.c. of toxicant solution sprayed from each nozzle at 12·5 p.s.i. during 5 sec. Each point the mean of 6 or 7 tests

KD_{50} (5 min.) : Py. I 0·060%; Py. II 0·015%
KD_{50} (10 min.): Py. I 0·029%; Py. II 0·009%

× Py. II 10 min. ○ Py. II 5 min. △ Py. I 10 min. ● Py. I 5 min.

FIG. 4 (right).—*Knockdown of* M. domestica *by 'Pyrethrin I' and 'Pyrethrin II' synergised by* 5 : 1 *ratio with piperonyl butoxide in the Kearns & March chamber*

Conditions as in Fig. 3. Each point the mean of 5–7 tests

KD_{50} (2·5 min.): Py. I 0·052%; Py. II 0·027%
KD_{50} (5 min.) : Py. I 0·023%; Py. II 0·012%

□ Py. II 5 min. ■ Py. I 5 min. ▽ Py. II 2·5 min. ▼ Py. I 2·5 min.

'Pyrethrin I' is synergised by piperonyl butoxide to a considerably higher extent than is 'Pyrethrin II'. Comparison of Figs. 3 and 4 indicates the factors of synergism for 'Pyrethrin I' and 'Pyrethrin II', at the 5 : 1 piperonyl butoxide/pyrethrins ratio, for the KD_{50} (5 min.) response to be 2·6 and 1·25 respectively. This parallels the much higher factor of synergism for Pyrethrin I than for Pyrethrin II, of piperonyl butoxide at the 8 : 1 ratio, found by Sawicki[28] for the kill of houseflies.

It is of interest that the mean value of the KD_{50} (5 min.) for 'Pyrethrin I' and 'Pyrethrin II' (Fig. 3) is 0·037 while that for the synergised products is 0·0175 (Fig. 4). Since these means should represent the KD_{50} (5 min.) concentrations for the unsynergised and synergised 1 : 1 mixture of 'Pyrethrin I' and 'Pyrethrin II', their ratio, 2·1, should give a value for the factor of synergism of piperonyl butoxide at the 5 : 1 ratio for the KD_{50} (5 min.) response of *pyrethrum extract*. This accords with the values of 2·1 and 2·0 for this parameter derived from Tables III and IV above and Figs. 3 and 4 of previous[1] work and with the values of 2·0 and 2·8 reported by Goodwin-Bailey[29] and Chadwick[30] respectively. Sawicki[31] reported the factor

Table XI

Kill of M. domestica *by 'Pyrethrin I' and 'Pyrethrin II' by topical application of measured drop*

30 Female flies (5 days old) per test: 2 × 0·1 μl. of the 0·3, 0·4, 0·5 and 0·6% toxicant solution applied to ventral surface under CO_2 anaesthesia: flies kept in jars supplied with 5% sugar solution and mortality count made after 24 h.: 6 replicates of each dose

Dose, μg./fly	% Mean kill Py. I	% Mean kill Py. II
0·6	21	18
0·8	66	58
1·0	86	82
1·2	97	94
LD_{50}, μg./fly from graph	0·74	0·78

Table XII

Kill of Tribolium castaneum *by 'Pyrethrin I' and 'Pyrethrin II' by topical application of measured drop*

30 Insects per test: 0·1 μl. of 0·4, 0·6, 0·8, 1·0, 1·2 and 1·4% solution of toxicant applied to ventral surface. Mortality count taken after 6 days: 6 replicates

Dose μg./insect	% mean kill Py. I	% mean kill Py. II
0·4	26	—
0·6	48	9
0·8	73	21
1·0	81	46
1·2	89	60
1·4	—	74
LD_{50}, μg./insect from graph	0·60	1·10

Table XIII

Kill of C. oryzae *by 'Pyrethrin I' and 'Pyrethrin II' by the dusted wheat method*

Dusts containing 0·5% pyrethrins on B.P. talc were prepared and an aliquot mixed with 50 g. of wheat. 50 weevils added to each bottle and mortality count made after 5 days. 3 Dose levels: 5 replicates

Dose, p.p.m. of wheat	% Mean kill Py. I	% Mean kill Py. II
6	22	10
8	53	28
10	79	43
LD_{50}, p.p.m. from graph	7·8	10·5

Table XIV

Recorded comparative activities of ' Pyrethrin I ' and ' Pyrethrin II '

Insect	Response	Formulation	Activity of ' Pyrethrin II ' (' Pyrethrin I ' = 1·00)	Reference
Blattella germanica	Kill	Water spray	0·8	13
Aphis rumicis	Kill	Water spray	0·1	14
Musca domestica	Kill	Kerosene spray	0·75	15
Aphis rumicis	Kill	Oil spray	1·0	16
Musca domestica	Kill	Aqueous acetone	1·0	16
Musca domestica	Kill	Kerosene	0·5	17
Musca domestica	KD_{50} (10 min.)	Kerosene spray	3·5	17
Blattella germanica	KD_{50} (30 min.) and kill	Kerosene spray	1·5	18
Tribolium castaneum	Kill	Water-oil spray	1·0	19
Tribolium castaneum	Kill	Aqueous alcohol	' Many times less active '	19
Musca domestica	Kill	Oil	0·25	20
Phaedon cochleariae	Kill	Acetone	0·4	21
Musca domestica	Kill	Oil	0·33	22
Musca domestica	Kill	Oil	0·25	23
Musca domestica	Knockdown		1·4	24
Musca domestica	Kill	Acetone	1·3	25

of synergism at the KD_{50} (10 min.) level for the 5 : 1 ratio piperonyl butoxide/pyrethrins to be 1·75.

For the kill of *Musca domestica, Triboleum castaneum* and *Calandra oryzae,* ' Pyrethrin II ' had 0·95, 0·55 and 0·74 times the activity of ' Pyrethrin I '.

Apart from low mammalian toxicity, *rapid* knockdown is the most highly prized property of the pyrethrins. The results of the present work suggest that advantage would accrue from the partial separation of ' Pyrethrin I ' and ' Pyrethrin II ' on the industrial scale. This can be easily effected[4] by attaching a second evaporator to the 12-in. still and carrying out the distillation with the two evaporators set at different temperatures, for example 160–180° and 230–240° respectively (compare the partial separation in Table VIII). The higher boiling fraction with diminished Py. I/Py. II ratio would have increased performance for the knockdown of houseflies without significant loss in lethal action ; the lower-boiling fraction with increased Py. I/Py. II ratio would have enhanced lethal activity against grain weevils, flour beetles and aphids.

Acknowledgments

The authors thank Dr. T. F. West and Mr. J. Olejniczak for valuable advice on the use of the 12-in. still, Miss Hardaker for the chemical analyses and the Chairman of the Pyrethrum Board (1960) for his stimulating interest.

Pyrethrum Board of Kenya
Nakuru, Kenya Republic

Received 30 October, 1964

References

1. Goldberg, A. A., Head, S., & Johnson, P., *J. Sci. Fd Agric.*, 1965, **16,** 43
2. Rius, A., Gispert, M., Martin, G. D., & Cardona, F., *An. real Soc. esp Fis. Quim*, 1953, **49B,** 633
3. Goodhue, L. D., & Haller, H. L., U.S.P. 2,358,392
4. Goldberg, A. A., & Smith, H. J., B.P. Appln. 7817/1959
5. Elliott, M., Olejniczak, J. S., & Garner, J. J., *Pyrethrum Post,* 1959, **3,** (2), 8
6. Cooper MacDougall & Robertson Ltd., B.P. 857,541
7. Olekniczak, J. S., & Rowe, T. W., *Le Vide,* 1960, (90), 469
8. ' Official Method of Pyrethrum Board of Kenya ', Sept., 1954
9. ' Official Methods of Analysis ', 7th edn., 1950 (Washington, D.C. : Ass. of Official Agricultural Chemists)
10. Smith, H. J., *J. Sci. Fd Agric.*, 1959, **10,** 260
11. Kearns, H. G. H., & March, B., *Soap, N.Y.*, 1943, **19,** (2), 101, 128 (Kettle, D. S., *Bull. ent. Res.*, 1949, **40,** 403)
12. Goodwin-Bailey, K. F., & Holborn, J. M., *Pyrethrum Post,* 1952, **2,** (4), 7
13. Gnadinger, C. B., & Corl, C. S., *J. Amer. chem. Soc.*, 1929, **51,** 3054
14. Tattersfield, F., *J. agric. Sci.*, 1929, **19,** 266
15. Gnadinger, C. B., & Corl, C. S., *J. Amer. chem. Soc.*, 1930, **52,** 3300

References (*cont.*)

[16] Hartzell, A., & Wilcoxon, F., *Contr. Boyce-Thomson Inst.*, 1936, **8**, 183

[17] Sullivan, W. N., Haller, H. L., McGovran, E. R., & Phillips, G. L., *Soap, N.Y.*, 1938, **14**, (9), 101

[18] McGovran, E. R., *Bull. U.S. Dep. Agric., Bureau of Entomology & Plant Quarantine*, 1941, No. E.544

[19] Martin, J. T., *Annu. Rep. Long Ashton Res. Sta.*, 1943, p. 62

[20] Gersdorff, W. A., *J. econ. Ent.*, 1947, **40**, 878; La Forge, F. B., & Barthel, W. F., *J. org. Chem.*, 1947, **12**, 199

[21] Ward, J., *Chem. & Ind.*, 1953, p. 586

[22] Incho, H. H., & Greenberg, H. J., *J. econ. Ent.*, 1952, **45**, 794

[23] Shen Chin Chang, *J. agric. Fd Chem.*, 1961, **9**, 390

[24] Sawicki, R. M., *Annu. Rep. Rothamsted exp. Sta.*, 1958,

[25] Sawicki, A. M., Elliott, M., Gower, J. P., Snarey, M., & Thain, E. M., *J. Sci. Fd Agric.*, 1962, **13**, 172

[26] Sawicki, R. M., & Thain, E. M., *J. Sci. Fd Agric.*, 1962, **13**, 292

[27] Sawicki, R. M., *Pyrethrum Post*, 1961, **6**, (2), 38; *Bull. ent. Res.*, 1961, **51**, 715

[28] Sawicki, R. M., *J. Sci. Fd Agric.*, 1962, **13**, 260

[29] Goodwin-Bailey, K. F., *Chem. & Ind.*, 1960, p. 700

[30] Chadwick, P., *Pyrethrum Post*, 1963, **7**, (1), 25

[31] Sawicki, R. M., *J. Sci. Fd Agric.*, 1962, **13**, 283

ISOLATION AND COMPOSITION OF LEAF PROTEIN FROM CERTAIN SPECIES OF INDIAN FLORA

By (Miss) A. VALLI DEVI, N. A. N. RAO and P. K. VIJAYARAGHAVAN

Analyses are reported on fresh leaves of some Indian flora for their % nitrogen, dry matter and extractable protein nitrogen contents, ash, fibre and the amino-acids, lysine and methionine.

There were wide variations in the nitrogen content of the leaves and the proportion of extractable protein nitrogen. The crude isolated materials have protein content varying from 34·2% in Tamarind to 77·4% in Sesbania. The crude isolates obtained from several sources had appreciable protein content and these warrant further studies as to their dietary value in deficient diets. The methionine and lysine contents of these proteins varied from 1·3% to 2·2% and 3% to 4% respectively.

Some of the proteins isolated are potentially useful for supplementation of cereal diets deficient in lysine and methionine.

Introduction

Protein foods of high nutritive value are in acute shortage among large segments of the world's population. This has necessitated many investigations for the development of suitable protein substitutes from various sources, including leafy vegetables. The possibility of utilising proteins obtained from green leaves as dietary supplements, to improve the quality as well as raise the protein level of deficient diet, was first suggested by British workers.[1–7] Chibnall and his co-workers[1–4] described in detail the earlier procedure used for the extraction and estimation of the nitrogen complex in certain leaf proteins. However, it was the classical studies carried out independently by Pirie[8–10] and Slade *et al.*[11] that led to the isolation of proteins on a pilot plant scale from green leaves such as sugarcane, cabbage, agave, lucerne and grass and their subsequent analysis for various nutrients, particularly their essential amino-acids. More recently, Byers[12] extracted proteins from leaves of several tropical plants from Ghana in quantities enough to justify their being considered as potential sources of protein and the plants were classified according to the extractability of protein nitrogen from leaf and the protein content of the isolates. The dietary utility of several isolated leaf proteins and their supplementary values have also been reported.[13–16]

In India, the first attempt to obtain proteins from leaves in a form suitable for human consumption was made by Guha and his associates in 1943, but few results were published. Subsequent reports[17–18] elucidated the conditions for maximum extraction of protein and highlighted the special features of leaf protein technology. Although leafy vegetables formed important ingredients in Indian dietary, information on their utility as dietary supplements is restricted to a limited number of leaves, such as amaranthus,[19, 20] trigonella[20] and lucerne,[21, 22] although the protein contents of some Indian leafy vegetables and grasses have been reported.[23, 24]

The results suggested the extension of the investigations to leaves of other Indian flora in order to provide sources of leaf proteins possessing appreciable amounts of essential amino-acids particularly lysine and methionine, and therefore having a possible supplementary value for diets. The composition of leaves of ten species of Indian flora as to their crude protein and methionine content were reported by Surinder Kaur & Vijayaraghavan.[25] This communication reports the results of further work on the isolation and analysis of leaf proteins from a number of sources.

Experimental

Crude proteins were isolated by the simple method of extraction suggested by Pirie,[10] from fresh leaves of a number of plants (see Table I). The leaves used were of medium maturity, collected at random from locally grown plants before the rainy season. Before use, the leaves were thoroughly cleaned with water to remove extraneous matter and their stems removed. Except in the case of coriander, drumstick, methi, sesbania and tamarind, the petioles of the leaves were also removed and the leaves were chopped into small pieces before use. About 100–150-g. lots of the leaves of mango, banana, sugarcane, Elephant grass, and 200–250-g. lots of other leaves were homogenised with water in a Waring Blendor for 10–15 min. The optimum quantity of water required for leaves of each variety was previously determined for each species and varied from 1½ to 3 times the weight of leaves. Only one extraction was carried out and the green homogenate was filtered through a fine cotton cloth to remove the fibrous-material. The filtrates from each lot of the same sample of leaves were bulked. 0·1N-Hydrochloric acid was added slowly with constant stirring to give a final pH of 4·5, when protein began to precipitate. The solution was heated to 60–70° to coagulate the protein and was then filtered.

The precipitate obtained at this stage did not keep well under normal ambient conditions. The crude material was repeatedly extracted with acetone to remove most of the water, lipoid material and colouring matter. The residual acetone was finally removed by drying the material at 60° under vacuum. The crude protein obtained had a grey to pale brown colour, with a powdery open texture and with no perceptible taste.

Analysis

Moisture was determined by drying the material to constant weight at 105° in oven; *nitrogen* by micro-Kjeldahl method with copper–selenium catalyst; and *ash* by incineration in a muffle furnace at 560° to constant weight. *Fibre content* was determined after digestion with hydrochloric acid and alkali in a water bath at 86° and filtration.

Lysine and methionine contents were determined by a microbiological procedure[26, 27] after acid hydrolysis of the crude protein by refluxing with 5N-hydrochloric acid for 24 h. *Lactobacillus mesenteroides P-60* was the organism used. The assay was carried out in triplicates of 5 dilutions of samples against standards similarly prepared in at least 8 dilutions. Methionine was also determined by the colorimetric method of McCarthy & Sullivan.[28]

The average values obtained for triplicate determinations are presented in Tables I and II.

Results

In Table I are shown the proportions of total protein in the isolated protein. The nitrogen contents of the dry leaves varied from 0·3% to 10% and % of extractable protein nitrogen ranged from 14% to about 60% of the total nitrogen. Although the total nitrogen contents of the leaves of coriander, amaranthus and chakothra were higher than that in other leaves when expressed on dry weight basis, the corresponding percentages of extracted protein nitrogen were only 14%, 14·3% and 28·4% respectively. On the other hand, for leaves of keerae, sugarcane, maize and mango, which had low contents of total nitrogen, the % of extracted nitrogen as crude protein was higher than that for most other plants. Correlation of nitrogen content with the proportion of extracted nitrogen of other plants showed that leaves of drumstick, methi, lucerne, soya and sesbania were potentially good sources of proteins, while those of bamboo, Elephant grass, banana, tamarind and coriander were poor sources for isolation of leaf protein.

Table II gives the analyses of the extracted protein material expressed on dry weight basis.

Table I

% of N in leaves correlated to N of crude isolates

Common name	Latin name	% of dry matter	N % on dry weight basis	% of protein N extracted	Remarks
1	2	3	4	5	6
Amaranthus	*Amaranthus gangiticus*	7·18	9·64	14·3	T, A
Bamboo	*Bambusa arundinacea*	45·37	1·65	17·5	M, E
Chakothra	*Artiplex rosea*	7·85	9·55	28·5	T, D
Coriander	*Coriander sativum*	11·15	10·14	14·0	T, B
Curry leaves	*Murraya koenigii*	29·70	3·36	17·4	M, B
Drumstick	*Moringa oleifera*	27·10	2·69	47·0	T, B
Elephant grass	*Pennisetum purpureum*	25·09	3·66	15·0	MT, C
Keerae	*Amaranthus compestris*	17·31	0·96	42·0	M, B
Lime	*Citrus aruntifolia*	34·05	2·29	22·0	MT, C
Lucerne	*Medicago sativa*	21·90	5·29	37·0	M, B
Mango	*Mangifera indica*	40·14	0·32	48·0	MT, E
Maize	*Zea mays*	27·81	0·59	39·0	M, E
Methi	*Trigonella faenumgraceum*	10·10	5·44	44·0	M, B
Banana	*Musa sapientum*	21·20	3·22	15·0	M, E
Sesbania	*Sesbania grandiflora*	26·55	4·21	25·5	M, B
Soya	*Glycine hispida*	17·12	3·97	31·0	M, B
Sugarcane	*Saccharum officinalis*	37·41	0·88	58·0	M, E
Tamarind	*Tamarindus indica*	34·89	2·43	14·0	T, B

A = No difficulty in mincing or making extract of the leaf
B = Plant had tough stems from which leaves are removed. No difficulty in extracting protein
C = Stems and petioles removed before mincing. No difficulty in extraction
D = Slight mucilage present, but no difficulty otherwise if stem and petioles removed
E = Leaves contain much fibrous material even after removing stems and petioles
M = Mature leaves 2–3 weeks old but not tough
MT = Mature and tough; 3–4 weeks old
T = Tender leaves 1–2 weeks old

Table II

Analyses of leaf protein isolates

Source of leaf protein	% on dry-weight basis			Amino-acid, g. (16 g. N)	
	Nitrogen	Ash	Fibre	Methionine	Lysine
Sesbania	12·9	5·2	2·9	1·57	3·80
Lucerne	12·2	3·1	5·9	1·24	1·85
Methi	11·5	4·4	2·7	1·26	3·78
Soya	11·3	2·5	3·3	0·85	2·87
Chakothra	10·4	6·9	1·3	1·48	2·89
Sugarcane	10·3	1·7	1·9	1·13	1·96
Coriander	9·3	5·8	2·1	1·42	3·21
Banana	8·7	7·4	1·7	2·18	4·35
Drumstick	8·6	4·5	1·2	2·04	3·46
Maize	7·6	2·5	1·3	1·76	2·28
Keerae	7·6	1·3	1·9	1·14	3·93
Bamboo	7·4	6·2	2·4	1·44	3·78
Amaranthus	7·4	7·5	1·6	1·74	3·20
Elephant grass	6·4	1·4	9·1	1·90	2·39
Curry	6·4	9·0	3·7	1·92	3·56
Lime	6·3	5·2	2·1	0·81	2·99
Mango	6·1	5·6	1·3	1·67	3·89
Tamarind	5·7	7·0	4·3	1·97	3·72

The nitrogen content of these isolated fractions varied widely from 5·7% in tamarind to 12·9% in sesbania. Four other preparations also contained appreciable protein (10–12%) in the extracted material. The fractions obtained from curry leaves, lime, mango and tamarind, particularly, were of low protein content. The ash contents of the isolated protein also showed wide variation from a value of 1·3% in keerae to a high value of 9·0% in curry leaves. (The separate elements present in the ash were not determined.) The fibre content of most of the leaf protein isolates was of the order of 1·3–4%, although the preparations from lucerne and Elephant grass had appreciably higher fibre contents.

The lysine and methionine contents are expressed as g. of amino-acid per 16 g. of protein N in Table II. The methionine contents of most of the proteins fell in the range 1·2% to 1·9%, except for lime and soya which gave lower values and banana and drumstick which gave higher

values. Most of the proteins had a lysine content in the range 3% to 4%. Banana, mango, sesbania, bamboo and methi isolates had slightly higher values, and grass, sugarcane and lucerne lower values.

Discussion

The protein contents of different leaves as well as the proportion of extractable protein have been shown to vary widely.[7] Reported values summarised up to 1958, indicate variation with respect to species, season of cutting, soil conditions and degree of maturity.[29] A considerable proportion of the total nitrogen of the leaves has been shown to be non-protein,[30, 31] while the structural proteins which form an integral part of cell structure mostly remain unextracted. The data presented here are based on estimations on duplicate collections of leaves, collected at random from local species of plants, before the monsoon season. However, the figures obtained for these leaves and their extracted protein did not show any significant variation and therefore average values have been given.

In recent studies on Ghana plants, Byers[12] attempted to classify these tropical plants into several categories based on the % extractable protein nitrogen and nitrogen contents of the isolates. The plants are broadly classified to six categories namely:

(*a*) more than 40% of total N extracted from leaves; more than 9% N in crude protein
(*b*) more than 40% of total N extraction from leaves; less than 9% N in isolates
(*c*) 20–40% of total N extracted from leaves; protein nitrogen of isolates above 9%
(*d*) 20–40% of total N extracted; below 9% N in isolates
(*e*) less than 20% of total N extracted; more than 9% N in isolates
(*f*) less than 20% of total N extracted; less than 9% N in isolates.

The number of plants studied here is only 18, but a similar classification would indicate that sugarcane, drumstick and methi come under category (*a*); keerae, mango and maize under category (*b*); lucerne, chakothra, sesbania and soya in category (*c*); lime in category (*d*); grass, banana and coriander in category (*e*); and tamarind, bamboo, curry and amaranthus in category (*f*). It may be mentioned here, that, while Byers extracted most plant leaves by centrifuging the mashed leaves and then extracted the fibrous residue with water, and precipitated the protein by heating the combined liquors at 80° and pH around 6, the present procedure involved one extraction with water and the precipitation of the protein by heating the extracts to 60–70° at pH 4·5.

The lysine and methionine contents of most of the leaf proteins, reported in literature[16, 29, 32, 33] fall within the range 5·2–7·1% for lysine and 1·3–2·7% for methionine. The present figures for methionine in most of the cases are within this range, whereas the values for lysine are slightly lower. Since the crude proteins were hydrolysed by refluxing for 24 h. before determination of amino-acids, partial loss of lysine by reaction with non-protein components cannot be excluded. The presence of some mineral elements in the crude protein may also have adversely affected the amino-acid during refluxing. The values for lysine obtained for the crude proteins from leaves of sugarcane, lucerne and Elephant grass do not compare with values reported by other workers,[29] perhaps due to difference in species or degree of maturity. Marked differences in the amino-acid make-up of leaf protein have been recently observed with variation in species[33] as well as in the degree of maturity of leaves.[29, 34] In the light of these reports, some variations in the amino-acid make-up of some leaf proteins are likely. It is, however, of interest to note that some of the leaf proteins studied, such as those of drumstick and banana, contained methionine in amounts comparable with that in the proteins of whole milk (2·8) or meat (2·0).[35] This would indicate that such leaf proteins may provide a means for improving the quality of pure vegetarian diet, which is usually characterised by marginal intake of essential amino-acids such as lysine and methionine.

Nutritional studies reported so far[13–16, 19–22] have indicated at least some supplementary values for most leaf proteins although grass proteins have been shown to possess a higher nutritive value than proteins from leguminous leaves.[36] Leaf proteins are generally considered to be adequately balanced with respect to their essential amino-acids[37] including lysine[38] and sulphur amino-acids.[7] A further stimulus has been provided by recent reports of Duckworth &

Woodham[14] where good nitrogen retention from protein isolates and gain in weight have been shown in chicks, rats and pigs and by Waterlow in Jamaican infants.[15] Waterlow's finding that, in infants recovering from malnutrition, the mean gain in weight on leaf protein-milk mixture was as good as that on milk at equal protein level, undoubtedly records the most encouraging report so far made on the dietary utility of leaf proteins.

Further work is obviously required to establish the supplementary value of the leaf proteins reported in this paper. Besides long-range nutritional studies, there is also need for improving the acceptability of the protein, for the characterisation of non-protein components such as glycosides, and the establishment of suitable combinations of leaf proteins which would complement each other in their amino-acid make-up.

Acknowledgment

The authors wish to express their thanks to the Deputy Chief Scientist for his interest and the Scientific Adviser to the Minister of Defence, for permission to publish this paper.

Defence Food Research Laboratory
Ministry of Defence
Mysore, India

Received 4 May, 1964; amended manuscript 24 July, 1964

References

[1] Chibnall, A. C., & Schvyver, S. B., *Biochem. J.*, 1921, **15**, 60
[2] Chibnall, A. C., *Biochem. J.*, 1922, **16**, 344, 599, 608
[3] Chibnall, A. C., & Grover, C. B., *Biochem. J.*, 1926, **20**, 108
[4] Chibnall, A. C., Miller, E. J., Hall, D. H., & Westall, R. G., *Biochem. J.*, 1933, **27**, 1879
[5] Lugg, J. W. H., *Biochem. J.*, 1938, **32**, 2114, 2123
[6] Tristram, G. R., *Biochem. J.*, 1939, **33**, 1271
[7] Crook, E. M., *Biochem. J.*, 1946, **40**, 197
[8] Pirie, N. W., *Chem. & Ind.*, 1942, p. 61
[9] Pirie, N. W., *Proc. Nutr. Soc.*, 1956, **15**, 154
[10] Pirie, N. W., *Food Manuf.*, 1957, **32**, 416
[11] Slade, R. E., Branscombe, D. J., & McGowan, J. C., *Chem. & Ind.*, 1945, p. 194
[12] Byers, M., *J. Sci. Fd Agric.*, 1961, **12**, 20
[13] Morrison, J. E., & Pirie, N. W., *J. Sci. Fd Agric.*, 1961, **12**, 1
[14] Duckworth, J., & Woodham, A. A., *J. Sci. Fd Agric.* 1961, **12**, 5; Duckworth, J., Hepburn, W. R., & Woodham, A. R., *ibid.*, p. 16
[15] Waterlow, J. C., *Brit. J. Nutr.*, 1962, **16**, 531
[16] *Nutr. Rev.*, 1963, **21**, 231
[17] Pal, P. R., & Guha, B. C., *Sci. & Cult.*, 1953, **18**, 597
[18] Chakravorthy, P. R., & Guha, B. C., *Proc. Symp. on Proteins*, (Mysore) 1960, p. 257.
[19] Deshpande, P. D., & Rao, M. V. R., *Indian J. med. Res.*, 1954, **42**, 77
[20] Kamath, S. H., & Kamala Schonie, *Indian J. med. Res.*, 1959, **47**, 93
[21] Sur, B. K., & Subramaniyan, V., *Indian J. med. Res.*, 1949, **37**, 319
[22] Sur, B. K., & Subramaniyan, V., *Curr. Sci.*, 1945, **23**, 188
[23] Theophilus, E., & Arulanantham, R., *Indian J. med. Res.*, 1949, **37**, 29
[24] Nath, N., & Das, N. B., *Indian J. vet. Sci. Animal Husb.*, 1953, **23**, 185
[25] Surinder Kaur, & Vijayaraghavan, P. K., *Curr. Sci.*, 1961, **30**, 298
[26] Dunn, M. S., Camien, M. N., Malin, R. B., Murphy, B. A., & Reiner, P. J., *Univ. Calif. Publ. Physiol.*, 1949, **8**, 293
[27] Barton-Wright, E. C., 'Microbiological Assay of the Vitamin B Complex and Amino-acids', 1952, p. 121, 142 (London: Pitman)
[28] McCarthy, T. E., & Sullivan, M. X., *J. biol. Chem.*, 1941, **141**, 871
[29] 'Proteins in Foods', Kuppuswamy, S., Srinivasan, M., & Subramaniyan, V., *Indian Counc. med. Res. Rep.*, 1958, No. 33, 221
[30] Swaminathan, M., *Indian J. med. Res.*, 1938, **25**, 847
[31] Kulkarni, L., & Sohonie, K., *Indian J. med. Res.*, 1956, **44**, 511
[32] Lugg, J. W. H., *Advanc. Protein Chem.*, 1949, **5**, 229
[33] Smith, A. M., & Agiza, A. H., *J. Sci. Fd Agric.*, 1951, **2**, 503
[34] Reber, E. F., & McVicar, R., *Agron. J.*, 1953, **45**, 17 (*Chem. Abstr.*, 1953, **47**, 8189)
[35] Block, R. J., & Booling, D., 'Amino acid Composition of Protein and Foods', 1951 (Springfield, Ill., U.S.A.: C. C. Thomas)
[36] Artmayer, J. H., Hernandez, G. R., & Cook, D. H., *J. agric. Univ. Puerto Rico*, 1938, **22**, 455 (*Chem. Abstr.*, 1939, **33**, 6980)
[37] Kelley, E. G., & Baum, R. R., *J. agric. Fd Chem.*, 1953, **1**, 680
[38] Lugg, J. W. H., & Weller, R. A., *Biochem. J.*, 1948, **42**, 408

SOCIETY OF CHEMICAL INDUSTRY

Monograph No. 8

THE QUALITY CONTROL OF FOOD

Comprising papers read at a Symposium organised by the Food Group and the Association of Public Analysts held in the Royal Society of Medicine, 1 Wimpole Street, London, W.1, 3–4 October, 1957

Price: **£1 0s. 0d.**

Price to Members: **15s. 0d.**

Orders should be sent to:

The Publications Department,
Society of Chemical Industry,
14 Belgrave Square,
London, S.W.1. (Tel: Belgravia 3681)

Journal of Applied Chemistry

The following papers are appearing in the February, 1965, issue

Corrosion of tube and pipe alloys due to polluted sea-water
By J. C. Rowlands

The effect of heat upon inorganic cements
By R. N. Rickles

The reaction of cyanuric chloride with gelatins and animal glues
By A. A. Leach

Dealkylation of aromatic hydrocarbons
By R. J. Harrisson, J. D. Brooks and K. McG. Bowling

The corrosion of stranded, tinned-copper cable in aqueous zinc chloride fluxes containing ammonium chloride
By G. B. Clarke and J. Golden

Extraction of uranium (VI) from nitric acid solutions by long-chain aliphatic amines
By Taichi Sato

Effect of surface treatment on the efficiency of stainless steel packings
By P. J. King and P. N. Walmsley

J. Sci. Fd Agric., 1965, Vol. 16, February

JOURNAL OF THE SCIENCE OF FOOD AND AGRICULTURE

CONTENTS

PAGE

Protein denaturation in frozen fish. IX.—The inhibitory effect of glycerol in cod muscle .. 65
By R. M. Love and M. K. Elerian

Determination of the water activity of some hygroscopic food materials by a dew-point method
By G. Ayerst 71

Acid-fume peeling of some food products 78
By K. Popper, F. S. Nury and W. L. Stanley

Studies on the nutritional value of foods treated with γ-radiation. I.—Effects on some B-complex vitamins in egg and wheat 81
By T. S. Kennedy

Insecticidal activity of pyrethrum extract and its four insecticidal constituents against house flies. VI.—Relative toxicity of pyrethrin I and pyrethrin II against four strains of house flies .. 85
By R. M. Sawicki and M. Elliott

Studies on the preparation, chemical composition and nutritive value of a spray-dried soya food suitable for feeding weaned infants 90
By S. R. Shurpalekar, Soma Korula, M. R. Chandrasekhara and M. Swaminathan

Specific and varietal differences in sodium and potassium in grasses 94
By G. ap Griffith, D. I. H. Jones and R. J. K. Walters

Lipids and protein denaturation in fish muscle 99
By June Olley and W. R. H. Duncan

Distillation of pyrethrum extract in a wiped-wall, falling-film, short-path still: separation of 'Pyrethrin I' and 'Pyrethrin II' and determination of their relative biological activities 104
By A. A. Goldberg, S. Head and (Mrs.) P. Johnson

Isolation and composition of leaf protein from certain species of Indian flora 116
By (Miss) A. Valli Devi, N. A. N. Rao and P. K. Vijayaraghavan

Abstracts **i-57—i-112**

Printed in Great Britain by Butler & Tanner Ltd, Frome and London