# THE CHROMOGEN METHOD FOR DETERMINING THE DIGESTIBILITY OF DRIED GRASS BY SHEEP

By J. DAVIDSON

The total collection method for determining digestibility has been compared with a ratio method that uses as a marker the absorption at wavelength 406 mμ of plant chromogens extracted from the food and faeces.

Results showed that when dried grass was fed to sheep the chromogen method gave low results when extracts were read at 406 mμ, and substantially correct results when read at 416 mμ. However, owing to the instability of the plant pigments involved, the plant chromogen method should be applied extensively only after comparative trials have shown that results for digestibilities agree with those found by reliable collection methods.

Estimates of the major fat-soluble plant pigments entering and leaving the alimentary tract showed that there were losses in all pigments during passage down the tract, but whereas losses of up to only 18% occurred in the carotenoids, losses of up to 62% were noted in the total fat-soluble tetrapyrroles and up to 87% in chlorophylls. The losses varied greatly from sheep to sheep.

## Introduction

Previous studies[1, 2] of fat-soluble pigments in the feed and corresponding rumen contents of sheep have shown that destruction of plant pigments takes place in the rumen, and it has been suggested[2] that an even greater destruction of labile plant pigments takes place in the abomasum and lower intestines. Because the 'chromogen' in the marker method of Reid *et al.*[3, 4] consists of a mixture of plant pigments, some of which are known to be labile, the following experiment was designed to gain information on the reliability of the chromogen method when used to determine the digestibility of roughage by sheep kept in metabolism crates. Digestibilities by the chromogen method were compared with those obtained by the total consumption–excretion method. A preliminary experiment with a single sheep showed that, on a poor-quality hay diet, low results were obtained by the chromogen method owing to poor recovery from the faeces of pigment material absorbing at 406 mμ. To find out whether some other wavelength might give results in closer agreement with those by the normal method, measurements of chromogen absorption were taken, in the following experiment, at wavelengths between 402 and 420 mμ at 2-mμ intervals.

A comparison of the amounts of the major plant pigments entering and leaving the alimentary tract of the sheep was also made to ascertain whether the degradation of plant pigments was sufficiently extensive to affect the reliability of marker methods based on plant chromogens, and whether any one plant pigment would provide a more reliable marker than the total pigments.

## Experimental

### *The diet*

A sufficient quantity of grass, grown and dried locally, and containing 12% of protein, was chopped and thoroughly mixed. Into each of 245 bags was weighed 350 g. of this chopped dried grass. Bag numbers 25, 50, 75, 100 and 150 were laid aside as samples of the whole, and the first 80 bags were fed during the experimental period.

### *Procedure*

Four Cheviot wethers were fed on 700 g. of the grass ration in two equal amounts twice daily at 9 a.m. and 5 p.m. for a 20-day preliminary and 10-day experimental period. For half the preliminary period the sheep were fed in individual pens and for the other half in metabolism crates of a standard design. During the experimental period in the same metabolism crates faeces were collected at 10 a.m. each day. Three of the animals had to be fitted with collection bags during the preliminary period because the faeces were too moist for easy removal from the crates. The fourth sheep gave well-formed faecal pellets throughout the experiment.

Each day one quarter of the weighed faeces was placed in an air-tight bottle and stored at $-20°$. At the end of the experiment all quarters were bulked, mixed and sub-samples taken for dry-matter estimation, chromogen extraction and chromatographic and spectrophotometric study of the major plant pigments.

These pigments, the chlorophylls, phaeophytins, carotene and xanthophyll were separated from the 85% acetone extracts of the grass feed and composite faeces by the method of Davidson.[1]

## Results

The total consumption–excretion results and digestibilities calculated from them are given in Table I. There was wide variation in the wet weights of faeces voided owing to wide variation

**Table I**

*Total collection results and derived digestibility percentages*

| Sheep no. | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Wt. on day 1, lb. | 85 | 93 | 93 | 74 |
| Wt. on day 30, lb. | 80 | 87 | 92 | 86 |
| Grass fed, g. | 7000 | 7000 | 7000 | 7000 |
| Dry matter of grass, % | 85·8 | 85·8 | 85·8 | 85·8 |
| Dry matter fed, g. | 6006 | 6006 | 6006 | 6006 |
| Faeces voided, g. | 4394 | 5745 | 4133 | 3634 |
| Dry matter of faeces, % | 32·71 | 24·81 | 34·77 | 40·85 |
| Dry matter voided, g. | 1437 | 1425 | 1437 | 1484 |
| Digestibility of dry matter, % | 76·1 | 76·3 | 76·1 | 75·3 |

in the moisture contents. However, the dry matter voided was remarkably constant, giving close agreement in the digestibilities of the dried grass. In Table II these digestibilities of dry matter are compared with digestibilities found by the chromogen method with wavelengths from 402 mμ to 420 mμ. The results by the chromogen method at 406 mμ were low by 3 to 7% but results at 416 mμ were in fairly good agreement with results by the total collection method. The results show that the most suitable wavelength on which to base calculations varies from 410 mμ for sheep 2 to 416 mμ for sheep 3 and 4.

**Table II**

*Digestibility percentages of the dry matter of dried grass by total collection and the chromogen method, using wavelengths from 402 to 420 mμ*

| Sheep no. | | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|
| Total collection method | | 76·1 | 76·3 | 76·1 | 75·3 |
| Chromogen method | mμ | | | | |
| | 402 | 70·5 | 72·2 | 67·8 | 68·2 |
| | 404 | 71·2 | 73·0 | 69·2 | 69·4 |
| | 406 | 72·1 | 74·0 | 70·6 | 70·8 |
| | 408 | 73·0 | 75·2 | 71·8 | 71·8 |
| | 410 | 73·9 | 76·2 | 73·5 | 72·9 |
| | 412 | 74·5 | 76·6 | 74·4 | 73·9 |
| | 414 | 74·7 | 77·2 | 75·3 | 74·5 |
| | 416 | 74·5 | 77·2 | 75·4 | 74·7 |
| | 418 | 74·2 | 77·0 | 75·1 | 74·4 |
| | 420 | 73·4 | 76·4 | 74·2 | 73·7 |

This variation in the most suitable wavelength was in part explained when the amounts of each pigment in the feed and faeces were compared on an undigested dry-matter basis (Table III). Table III shows that, although there were losses in total carotenoids during passage through the alimentary tract, these were small compared with the high losses of chlorophyll-*a* and -*b*, and even phaeophytin-*a*. Further, the losses varied greatly from sheep to sheep. Of the total fat-soluble tetrapyrroles entering the alimentary tract some 40, 50, 62 and 56% were lost during passage through the tracts of sheep 1, 2, 3 and 4 respectively.

An indication that losses of the major fat-soluble pigments were likely to be high in the alimentary tract was given early in the separation procedures, when it was noted that 25–35% of the pigments extracted by acetone from faeces, and absorbing at 660 mμ, could not be transferred from the acetone to diethyl ether for chromatographic analysis. These losses were probably caused by degradation products which were more soluble in dilute aqueous acetone than in diethyl ether.

**Table III**

*Estimates of the individual fat-soluble plant pigments in the roughage fed and faeces voided, and the calculated losses during passage through the alimentary tract*

| | | Dry-matter basis | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Chlorophyll-*a* | Chlorophyll-*b* | Phaeophytin-*a* | Phaeophytin-*b* | Total fat-soluble tetrapyrroles | Carotene | Xanthophyll | Total carotenoids |
| Dried grass: average found, mg./100 g. | | 59 | 46 | 172 | 12·0 | 289 | 19·3 | 9·7 | 29·0 |
| Faeces: | | | | | | | | | |
| Sheep 1 | Calculated,* mg./100 g. | 247 | 193 | 717 | 50 | 1207 | 81 | 41 | 122 |
| | Average found, mg./100 g. | 47 | 45 | 558 | 68 | 718 | 75 | 35 | 110 |
| | Loss, % | 81 | 77 | 22 | −36 | 40 | 7 | 15 | 10 |
| Sheep 2 | Calculated,* mg./100 g. | 249 | 194 | 723 | 50 | 1216 | 81 | 41 | 122 |
| | Average found, mg./100 g. | 45 | 35 | 484 | 45 | 609 | 83 | 37 | 120 |
| | Loss, % | 82 | 82 | 33 | 10 | 50 | −3 | 10 | 2 |
| Sheep 3 | Calculated,* mg./100 g. | 247 | 193 | 717 | 50 | 1207 | 81 | 41 | 122 |
| | Average found, mg./100 g. | 32 | 34 | 345 | 42 | 453 | 74 | 33 | 107 |
| | Loss, % | 87 | 82 | 52 | 16 | 62 | 9 | 20 | 12 |
| Sheep 4 | Calculated,* mg./100 g. | 239 | 186 | 694 | 48 | 1167 | 78 | 39 | 117 |
| | Average found, mg./100 g. | 39 | 33 | 398 | 40 | 510 | 71 | 33 | 104 |
| | Loss, % | 84 | 82 | 43 | 17 | 56 | 9 | 15 | 11 |

* Calculated from digestibility of dry matter by collection method as follows:

$$\text{Calculated figure} = \frac{\text{mg./100 g. in grass} \times 100}{100 - (\%\ \text{digestibility of grass})}$$

## Conclusion and discussion

Earlier studies[2] have shown that degradation of chlorophylls occurs in the rumen. The present investigation shows clearly that losses of phaeophytins, carotene and xanthophyll as well as chlorophylls occur in the alimentary tract as a whole, and that the extent of these losses varies from animal to animal on the same diet. There would be little or no advantage in using any one of the plant pigments as a marker.

The losses are extensive for the fat-soluble tetrapyrroles as a whole and chlorophylls in particular. However, in an acetone extract of faeces the over-all loss of absorption in the 400–420-m$\mu$ region of the spectrum due to degradation of plant pigments is apparently compensated by the absorption of resultant degradation products and pigmented compounds from intestinal secretions and microflora that are soluble in 85% acetone.

In this experiment digestibilities based on chromogen absorption at the wavelength 406 m$\mu$ as suggested by Reid *et al.*[3, 4] are decidedly low, whereas those based on absorption at 416 m$\mu$ are substantially correct. Rogozinski[5] suggested that variations in chlorophyll degradation that he observed in sheep fed on different diets were caused by varying acidity of the chyme due to different amounts of available carbohydrate in these diets. Dietary influences of this kind could account for variations found in the most suitable wavelength for use in digestion trials involving plant chromogen techniques.

In view of the extensive degradation of plant pigments in the alimentary tract it seems likely also that the proportion of most labile to least labile pigments in the forage or fodder will affect the extent of total degradation obtained, and thereby cause a variation in the most suitable wavelength at which to measure 'chromogen' absorption and on which to base digestibility calculations. This conclusion agrees with the finding of Smart *et al.*[6] that the so-called isobestic point varies between 390 and 425 m$\mu$, according to the roughage and species of animal.

As Irvin *et al.*[7] have already suggested, an improved marker-method of more general application might be evolved if an acid or alkali treatment of the extract from roughage and faeces could alter all tetrapyrrole pigments to the same equilibrium mixture. However, the present results suggest that it would be imprudent to apply the plant chromogen method

extensively until comparative trials on feeding the roughage in question to the species being studied have shown that at a certain suitable wavelength between 400 and 420 mμ substantial agreement is given with reliable collection methods.

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# PHYSICOCHEMICAL STUDIES ON THE APPLICATION OF INSECTICIDES TO SHEEP FLEECE. V.*—The Influence of Carbon-chain Length and Halide Ion of Cationic Wetting Agents on their Reaction with Natural Fleece

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The influence of variation in chain length on the reactions between fleece and cationic wetting agents has been studied, with particular reference to octadecyl-, cetyl-, tetradecyl- and dodecyl-pyridinium chlorides. Reactions with cetyltrimethylammonium chloride closely resemble those with cetylpyridinium chloride. The inactivation of the wetting agents, and change in weight of the fleece samples, have been determined over ranges of concentration, and the two properties correspond closely in all instances. With octadecyl and cetyl compounds considerable inactivation occurs, but with the tetradecyl and dodecyl compounds the interfacial activity of the wetting agent is enhanced; this is attributed to the increase in the solubility of the long-chain cation–suint anion complex with decrease in cation chain-length. At $C_{14}$ and below, the flocculation of suspended matter, which is typical of the longer chains, does not occur. All the curves may be interpreted on the basis of the $\alpha$, $\beta$ and $\gamma$ mechanisms discussed in earlier Parts. The effect of addition of sodium chloride on reactions with cetylpyridinium chloride is considered. Dodecyl- and tetradecyl-pyridinium compounds have been used to compare the properties of the various halides. The change in properties produced by one step in the series $Cl^- \rightarrow Br^- \rightarrow I^-$ is approximately equivalent to an increase of two carbon atoms in the cation chain-length.

Previous papers in this series have described the reactions that take place between cationic wetting agents and natural fleece, with particular reference to cetylpyridinium chloride; considerable decrease in the interfacial activity of these solutions was observed under almost all conditions. The various reactions already outlined are characteristic of cationic wetting agents, and will occur irrespective of the carbon-chain lengths involved, but the extent to which they occur, and the influence of the reaction products on the interfacial activity of the solution, will be determined by a number of factors, among which the carbon-chain length and the halide ion of the wetting agent are important. For example, decrease in the length of the cationic carbon-chain will increase the solubility of the cation–suint anion complex, and the interfacial activity of the solution; it will also vary the critical concentration for micelles, and thus the concentrations at which the various mechanisms operate. Since it is not practicable to alter the temperature of a dip-bath, the range of compounds studied is limited to those having a sufficient solubility at room temperature.

* Part IV: *J. Sci. Fd Agric.*, 1954, **5**, 139

## Materials and experimental method

*Alkylpyridinium chlorides.*—These were prepared from pure, dry pyridine, and the alkyl chlorides by the method of Knight & Shaw.[1] The alkyl chlorides were purified by low-pressure fractional distillation, using an electrically heated column, three feet long, packed with glass helices and fitted with a total reflux partial take-off stillhead. The fractions collected were: dodecyl chloride, b.p. 145–146°/20 mm.; tetradecyl chloride, b.p. 147–149°/7 mm.; cetyl chloride, b.p. 156–158°/2 mm.; octadecyl chloride, b.p. 176–179°/2 mm.

*Cetyltrimethylammonium chloride.*—This was prepared by a modification of the method of Westphal & Jerchal.[2] Equivalent quantities of cetyl chloride and trimethylamine, dissolved in a small volume of absolute alcohol, were sealed in a resistance-glass tube and heated in a Carius furnace at 120° for 5 hours. On being cooled, the quaternary salt crystallized. It was isolated by dissolving the solid in the minimum quantity of alcohol, adding dry ether and cooling. The crystals were washed with ether, and recrystallized (m.p. 70°). The product was slightly deliquescent; the corresponding tetradecyl and dodecyl compounds were deliquescent.

*Alkylpyridinium bromides and iodides.*—Dodecylpyridinium bromide was prepared by interaction of the chloride with ammonium bromide in ethanol solution. Dodecylpyridinium iodide and tetradecylpyridinium bromide were prepared by treating aqueous solutions of the chlorides with the appropriate potassium halide, and crystallizing at 0°. The products were purified by recrystallization from alcohol and ether.

*Fleece samples.*—It has been shown in Part III[3a] that root flank and tip flank samples represent two extremes of fleece behaviour in these systems. For this reason, most experiments have been carried out with both root flank and tip flank samples; the whole of the flank fleece of a Scotch Blackface ewe (born 1947, shorn 1951) was used for this purpose. The root and tip sections were separated as described in Part II,[3b] the two composite samples being thoroughly mixed. Relevant properties are given below.

| Sample | Grease, % | Suint, % | Surface area, cm.²/g. |
|---|---|---|---|
| Root flank | 9·3 | 18·2 | 456 |
| Tip flank | 3·4 | 3·0 | 513 |

*Inactivation measurements.*—The term 'inactivation' refers to a decrease in the interfacial activity of the solution, and the term 'activation' to an increase in this activity. Because of the large magnitude of the activation observed in some of these experiments, and because it is necessary to compare directly the effects produced by compounds of differing molecular weights, it has been necessary to change the units employed. In this paper, inactivation (or activation) is expressed in terms of the apparent change in molar concentration ($\Delta$M) produced by the immersion of a 1-g. sample of fleece in 50 ml. of solution. The apparent final concentration was determined from the interfacial tension of the solution; for this purpose it was necessary to dilute the solution below the critical concentration for micelles. It should be stressed therefore that these units are hypothetical, since they do not necessarily represent the true change in concentration of wetting agent; the results show, however, that this treatment provides an excellent means of following the changes taking place in these systems. All isotherms given below were determined after 30 minutes' immersion of the fleece sample.

*Weight-loss measurements.*—The change in weight of the fleece samples during immersion was determined by weighing the samples after drying and conditioning in air; these measurements were carried out on the actual samples used for the inactivation measurements, so that the weight-loss and inactivation curves may be directly related.

## Effect of chain length

Fig. 1 shows 30-minute isotherms for root flank fleece, using alkylpyridinium halides of chain length $C_{12}$ to $C_{18}$. There is a pronounced and progressive chain-length effect, and for chain lengths of $C_{14}$ and $C_{12}$ the interfacial activity of the solution is considerably enhanced over the full range of concentration. In view of the practical importance of this observation, the chain lengths have been considered individually.

*The octadecylpyridinium cation.*—This isotherm is a straight line throughout (A, Fig. 1). The $\alpha$ mechanism completely submerges the $\beta$ and $\gamma$ mechanisms. Virtually all the cationic agent is removed from solution at every concentration used, and the final concentration never approaches the critical value, even though this is as low as 0·00038M. As the initial concentration is increased, the solutions become very turbid, owing to the formation of an insoluble

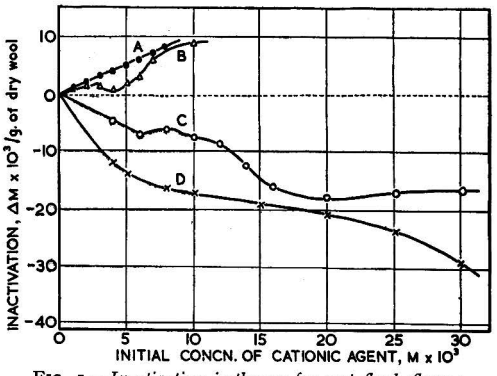


FIG. 1.—*Inactivation isotherms for root flank fleece: effect of chain length of wetting agent*

Curve A: octadecylpyridinium chloride
" B: cetylpyridinium chloride
" C: tetradecylpyridinium chloride
" D: dodecylpyridinium chloride

complex between the $C_{18}$ cation and suint. This does not redeposit on the fibres over initial concentrations up to 0·008M. Removal of grease does not take place, since loss in weight of the fleece during immersion is near, and in most cases slightly less than, the suint content. Results with tip flank samples are somewhat different, but consistent; they are compared in Table I.

**Table I**

| Initial concentration, M × 10³ | Inactivation, ΔM × 10³ | | Weight loss, % | |
|---|---|---|---|---|
| | Root fleece | Tip fleece | Root fleece | Tip fleece |
| 0·25 | 0·24 | 0·24 | 20·8 | — |
| 0·5 | 0·48 | 0·48 | 19·4 | 4·0 |
| 1·0 | 0·97 | 0·97 | 17·8 | — |
| 2·0 | 1·9 | 1·9 | 18·8 | 5·6 |
| 3·0 | 2·9 | 2·8 | 18·1 | — |
| 4·0 | 3·7 | 3·5 | 15·4 | — |
| 5·0 | 4·8 | 4·5 | 16·7 | — |
| 6·0 | 5·7 | 4·9 | 10·4 | 5·6 |
| 8·0 | 7·9 | 5·8 | — | 7·4 |

Over the first half of the concentration range the inactivation values for tip samples follow exactly those for the root samples. Beyond a concentration of 0·003M we may consider that the limited amount of suint available on the tip samples (3·0%) is all converted into insoluble cation–anion complex, and that afterwards the concentration of octadecylpyridinium chloride begins to increase. Some grease is then removed, which exposes wool protein to reaction with the wetting agent. At and above initial concentrations of about 0·0035M, the inactivation values for tip samples fall below those for root samples by an amount in excess of the critical concentration, so that conditions are appropriate for grease removal and protein reaction. In micellar solutions of $C_{16}$ compounds and those of shorter chain length, these two processes are associated with considerable activation, whereas with $C_{18}$ the decrease in inactivation is only slight. We therefore presume that the products of interaction of the $C_{18}$ compound with either grease or protein are too insoluble to display any considerable surface activity.

*The cetylpyridinium cation.*—This cation was dealt with fully in Parts II and III, and has therefore been taken as the standard for purposes of comparison.

*The tetradecylpyridinium cation.*—Fig. 2 shows activation isotherms and weight-loss curves for both root flank and tip flank fleece. The most striking feature of these curves is that the solutions are activated throughout the whole range of concentration. This change in properties must be related to the variation in the solubility of the long-chain cation–anion complex with chain length. The interfacial activity of long-chain ions is increased by reduction in the charge density of the associated gegenion. If this gegenion is a second long-chain ion, its charge density is so low that the interfacial activity of the adsorbing ion is greatly enhanced.

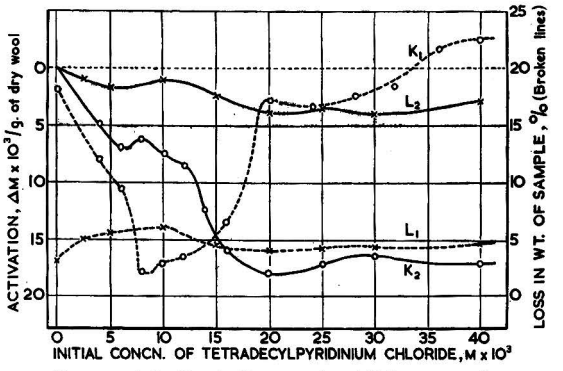


FIG. 2.—*Activation isotherms and weight-loss curves for tetradecylpyridinium chloride*
Curves $K_1$ and $K_2$: root flank fleece
" $L_1$ and $L_2$: tip flank fleece

A parallel investigation has been carried out in which solutions of these cationic agents were titrated against solutions of salts of the long-chain fatty acids, and the change in interfacial activity of the solution was followed. This work will be described in detail elsewhere, but the results may be broadly summarized in the following diagram, where Py represents pyridinium:

| | $C_7H_{15}\cdot CO_2^-$ Na+ | $C_9H_{19}\cdot CO_2^-$ Na+ | $C_{11}H_{23}\cdot CO_2^-$ Na+ |
|---|---|---|---|
| $C_{18}Py^+$ $Cl^-$ | − | −− | |
| $C_{16}Py^+$ $Cl^-$ | + | − | −−− |
| $C_{14}Py^+$ $Cl^-$ | +++ | ++ | − |
| $C_{12}Py^+$ $Cl^-$ | +++ | +++ | ++ |

The minus signs indicate a decrease, and the plus signs an increase, in the interfacial activity of the solution of cationic agent on addition of the anionic agent, and the number of such signs illustrate the relative extent of these effects. The fleece properties summarized in Fig. 1 show that the change from inactivation to activation occurs between cationic chain lengths of $C_{16}$ and $C_{14}$. It follows from the diagram shown above that the action of fleece on the solution of cationic agent simulates that of a solution containing $C_9H_{19}\cdot CO_2^-$ ions. Freney[4] deduced that the mean molecular weight of the potassium salts of long-chain fatty acids contained in suint was 200. Since the molecular weight of the compound $C_9H_{19}\cdot CO_2K$ is 210, the present work leads to a similar conclusion, though it is based on an entirely different approach.

The higher content of suint on the root samples (Fig. 2) produces much greater activation than is observed for the tip samples, but in each instance there is a close relation between the weight-loss curves and the activation isotherms. The concentration range can be separated into $\alpha$, $\beta$ and $\gamma$ sections, as with cetylpyridinium chloride (Part III). In the $\alpha$ range, visual examination of the solution showed that more of the complex is formed than will dissolve in the solution available. Adsorption and absorption on the fibres is pronounced, as indicated by the rapid fall of the initial part of curve $K_1$; the presence of long-chain gegenions in the solution is responsible for this, and for the high activation, whereas with the cetyl compound no such gegenions are present in solution. The $\beta$ mechanism is not greatly influenced, since grease is again removed and surface-active material returned to the solution. The $\gamma$ mechanism is, however, quite different, since the presence of the complex prevents any considerable flocculation of suspended material; both the weight loss and the activation remain high.

*The dodecylpyridinium cation.*—Curve D, Fig. 1, shows that the various effects to which the activation of solutions of tetradecylpyridinium chloride was attributed are even more pronounced for the dodecyl compound. Although the $C_{12}$ cation–suint anion complex has, in itself, a lower interfacial activity than the corresponding $C_{14}$ complex, it has a higher solubility, and this leads to more rapid activation in dilute solutions. The high interfacial activity of the solution masks the breaks in the curves that occur with longer chain-lengths. The weight-loss curve is similar in form to curve $K_1$, Fig. 2; the curve does not fall so rapidly, and reaches a minimum at 8% weight loss instead of 2%. Afterwards the loss in weight

increases, and remains about 20% up to concentrations of 0·06M, so that no flocculation occurs.

*Comparison between cetylpyridinium and cetyltrimethylammonium cations.*—Among the most important cationic agents in industrial use are the long-chain pyridinium and the long-chain substituted-ammonium halides. The behaviour of cetyltrimethylammonium chloride solutions in contact with fleece is compared with cetylpyridinium chloride in Fig. 3, for samples of both root and tip fleece. The isotherms for the two compounds show very similar features. Those for cetyltrimethylammonium chloride in the $\alpha$ range are also straight lines, and, although they cover a smaller range of concentration, the break is still related to the critical concentration. The behaviour in the $\beta$ range is almost identical, but the fact that curves P and R do not diverge so much as curves M and N in the $\gamma$ range indicates that flocculation occurs to a less extent in solutions of cetyltrimethylammonium chloride.

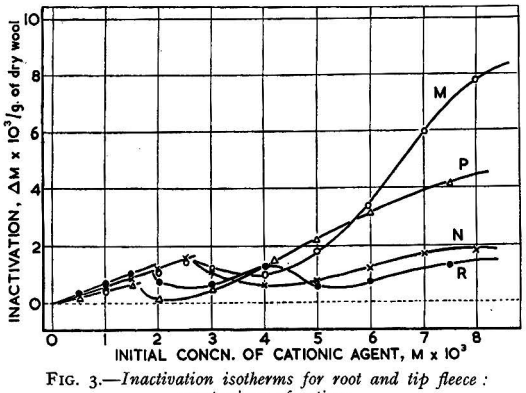


FIG. 3.—*Inactivation isotherms for root and tip fleece: comparison of cations*

| Curve | Wetting agent | Wool sample |
|---|---|---|
| M | cetylpyridinium chloride | root flank |
| N | cetylpyridinium chloride | tip flank |
| P | cetyltrimethylammonium chloride | root flank |
| R | cetyltrimethylammonium chloride | tip flank |

*Effect of addition of simple electrolyte.*—The interfacial activity of cationic wetting agents is influenced profoundly by the presence of simple electrolytes (see Fig. 5, Part II). Weight-loss curves and inactivation isotherms in solutions containing a high concentration (0·007M) of sodium chloride have therefore been determined for root flank fleece, and the results are compared in Fig. 4 with curves obtained in the absence of sodium chloride. In measuring the final concentrations from interfacial tension data, those solutions above the critical concentration were diluted with 0·007M-sodium chloride solution, and the concentration was determined from a tension–concentration curve appropriate to this solution. Curves $T_1$ and $T_2$ resemble those for cetylpyridinium chloride alone, and the $\alpha$, $\beta$ and $\gamma$ ranges can still be detected in curve $T_2$, but the increase in surface activity due to addition of sodium chloride introduces features that are reminiscent of the results from the experiment with octadecyl-pyridinium chloride (Table I). Thus, the $\beta$ range is almost submerged (curve $T_2$) and cannot be detected in curve $T_1$, although $\gamma$-range flocculation and redeposition still occur. Curve $T_1$ also shows an unusual feature, which has only been observed elsewhere with octadecyl-pyridinium chloride (Table I, column 4), i.e. the rather sudden decrease in weight of the sample in very low concentrations of wetting agent.

**Influence of halide ion**

Tension measurements have shown that the interfacial activity of the bromides and the iodides is much greater than that of the chlorides, and, since the bromides are in common industrial supply, a study has been made of the influence of halide ion on reactions between fleece and wetting agents. When the addition of fleece to a solution of a surface-active agent produces pronounced inactivation, successive additions of such samples will eventually lead to complete removal of the agent from the solution. This undesirable feature is characteristic of cetyl- and octadecyl-pyridinium compounds, but the dodecyl- and tetradecyl-pyridinium compounds were considered to be worthy of further study, in view of the considerable activation of their solutions by fleece.

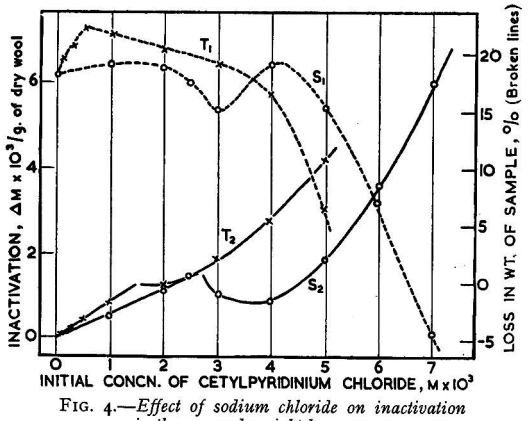


FIG. 4.—*Effect of sodium chloride on inactivation isotherms and weight-loss curves*

Curves $S_1$ and $S_2$: cetylpyridinium chloride alone
„ $T_1$ and $T_2$: cetylpyridinium chloride in 0·007M-sodium chloride solution

Figs. 5 and 6 show the 30-minute isotherms for dodecylpyridinium and tetradecylpyridinium halides respectively, with samples of root flank fleece. The change from dodecylpyridinium chloride to iodide produces an effect as great as an increase in chain length from $C_{12}$ to $C_{16}$. Tetradecylpyridinium iodide is not sufficiently soluble to permit measurement of its isotherm at room temperature, but the same large difference between the chloride and bromide is observed.

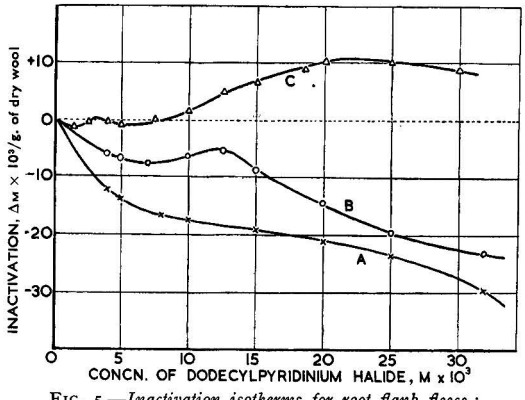


FIG. 5.—*Inactivation isotherms for root flank fleece: influence of halide ion*

Curve A: dodecylpyridinium chloride
„ B: dodecylpyridinium bromide
„ C: dodecylpyridinium iodide

This pronounced effect of halide is in accord with the surface chemistry of these compounds. A separate study (to be described in detail elsewhere) of the surface properties of these solutions in the absence of fleece show that the interfacial activity increases markedly, and the critical concentration for micelles is lowered, as the halide is varied from fluoride to iodide. The slopes of the interfacial tension curves show that the long-chain cation is more strongly adsorbed with increase in the size of the halide ion, and we consider that the degree of adsorption of the cation is related directly to the charge density of the halide ion, which decreases from fluoride to iodide. The distribution of long-chain cations between surface and bulk solution depends upon their relative energies in these two states; the energy of the cation is influenced by the charge density of the associated gegenions, though not to the same extent in the two states. The variation in the charge density of the free halide ions is offset somewhat in solution by varying degrees of hydration, but the effective charge densities are

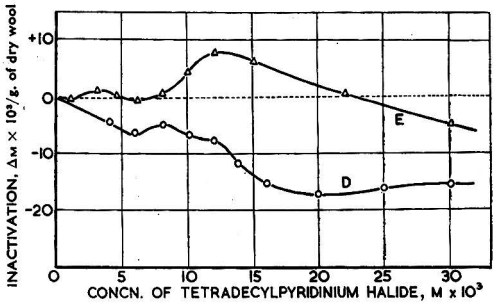


FIG. 6.—*Inactivation isotherms for root flank fleece: influence of halide ion*
Curve D: tetradecylpyridinium chloride
„ E: tetradecylpyridinium bromide

still sufficiently different to cause considerable variation in the surface properties of the solutions. Studies on the titration of cationic against anionic surface-active agents have shown that long-chain anions cause much greater adsorption of long-chain cations than do the iodide ions; this is in keeping with the very low charge density of long-chain anions. When fleece is immersed in solutions of the alkylpyridinium halides, the adsorption of the cation is determined by both the suint anions and the halide ion. In such a system the influence of the long-chain anion on cation adsorption outweighs that of the halide ion, so that the true halide effect is diminished, although Figs. 5 and 6 show that it is still important. The long-chain cation–anion titrations have also shown that the solubility of these complexes decreases as the halide ion is changed progressively from fluoride to iodide, but the decrease in solubility is not so great as that produced by increase in the chain length of the cation.

On these considerations alone, it would appear that the relative position of the curves in Figs. 5 and 6 is the reverse of that expected, but the curves are readily interpreted when the nature of the solid surface at which adsorption is taking place is taken into account. These curves show changes in surface activity, and the actual surface activity of the solution does not determine their position. When the chloride ion is replaced by bromide (cf. curves A and B, Fig. 5) the surface activity of the solution, and the degree of adsorption at the wool surface, is increased. In the $\alpha$ concentration-range adsorption is irreversible at this surface, since long-chain ions originally adsorbed become absorbed in, or chemically bound to, the grease layer in quantities of about 50 times that required for a monolayer (Part II[3]). As a result of the increase in surface activity, more cationic agent is removed from solution; this, coupled with the decrease in solubility of the long-chain anion–cation complex in the presence of bromide, leads to less activation of the solution than occurs in the presence of chloride. The relative position of the bromide and iodide curves (B and C, Fig. 5) is similarly accounted for.

The processes that determine the change in position and shape of the dodecylpyridinium halide isotherms with increasing size of halide ion are therefore virtually the same processes that determine the variation in the isotherms with increasing chain length. Thus the dodecylpyridinium iodide isotherm shows the $\alpha$, $\beta$ and $\gamma$ concentration-ranges as clearly as do the tetradecylpyridinium bromide isotherm (Fig. 6) and the cetylpyridinium chloride isotherm (Fig. 4). Broadly speaking, the change in the isotherm produced by one step in the series $Cl^- \rightarrow Br^- \rightarrow I^-$ is equivalent to an increase of two carbon atoms in the cation chain-length.

The 30-minute isotherms and weight loss curves for dodecylpyridinium bromide are given in Fig. 7. The general shape and position of the curves for both root and tip fleece samples resemble closely those for tetradecylpyridinium chloride (Fig. 2). The only significant difference in behaviour results from the differing degrees of flocculation in the two instances. The flocculating power of the wetting agent increases more rapidly with increase in chain length than with size of halide ion, so that the steady increase in activation and weight loss observed at higher concentrations of dodecylpyridinium bromide is arrested by some flocculation for tetradecylpyridinium chloride.

The curves for dodecylpyridinium iodide (Fig. 8) may be compared in a similar manner with those for tetradecylpyridinium bromide (Fig. 9). In each case the weight-loss curves reflect the changes in direction of the isotherms; when allowance is made for the differing micelle concentrations in the two instances, the two weight-loss curves for root fleece show a remarkable resemblance. The root-fleece isotherms are also similar; a higher micelle con-

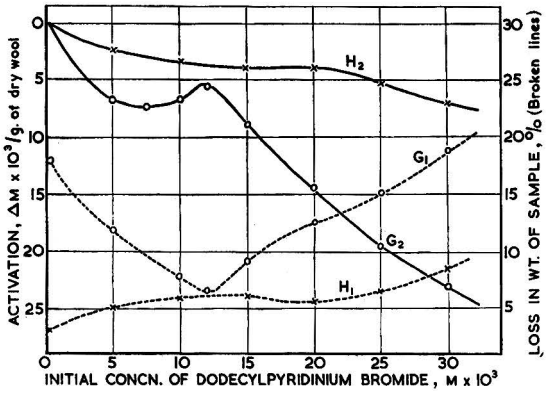


FIG. 7.—*Activation isotherms and weight-loss curves for dodecylpyridinium bromide*
Curves $G_1$ and $G_2$: root flank fleece
„ $H_1$ and $H_2$: tip flank fleece

centration for dodecylpyridinium iodide results in a shift of the breaks in the curves to higher concentrations than with tetradecylpyridinium bromide, so that the curves in Fig. 8 do not cover the whole of the comparable concentration range. At higher concentrations than those shown in Fig. 8 cation–protein reactions are more pronounced, and the isotherm $S_2$ shows a fairly steep fall, resembling curve $K_2$ (Fig. 9).

*Relation between micelle formation and grease removal*

It was shown in Part III[3] that, with cetylpyridinium chloride solutions, grease removal from the wool fibres only occurred when the concentration of the solution exceeded the critical concentration for micelle formation, and it was suggested that the grease was removed by a process of solubilization in the micelles. It is now possible to see how far this property is common to other wetting agents.

The critical concentrations given in Table II were determined at 18·5°. The values in column 2 are the final concentrations of the solutions (in contact with fleece, and determined from interfacial tensions), not the initial concentrations. The compounds marked with an asterisk are those that show inactivation, and in each instance the concentration at which grease removal begins is in close agreement with the critical concentration of the wetting agent itself. With the remaining compounds there is no agreement; here considerable

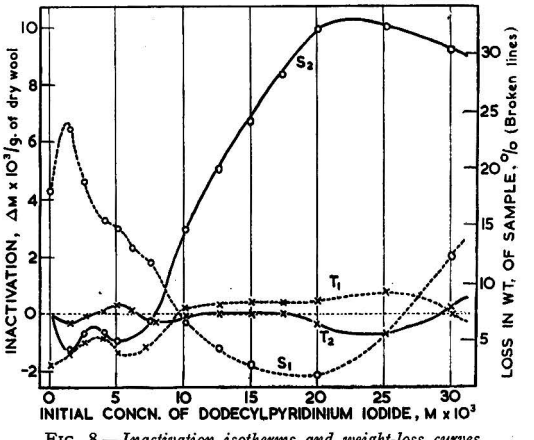


FIG. 8.—*Inactivation isotherms and weight-loss curves for dodecylpyridinium iodide*
Curves $S_1$ and $S_2$: root flank fleece
„ $T_1$ and $T_2$: tip flank fleece

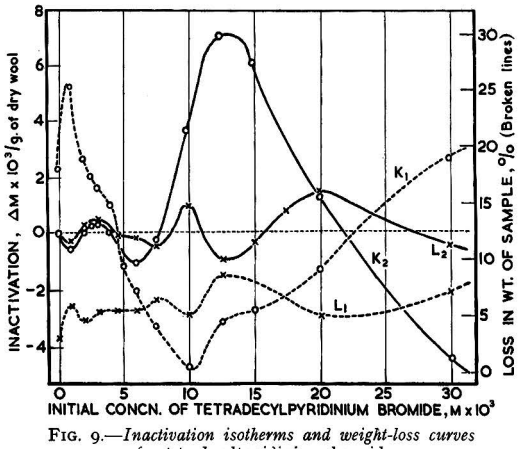


FIG. 9.—*Inactivation isotherms and weight-loss curves for tetradecylpyridinium bromide*
Curves $K_1$ and $K_2$: root flank fleece
„ $L_1$ and $L_2$: tip flank fleece

activation of the solution is caused by the higher solubility of the cation–suint anion complex, and this in turn alters the critical concentration of the solution.

**Table II**

| Wetting agent | Apparent concn. (molar) at which grease removal begins | Critical concn. (molar) from interfacial tensions |
|---|---|---|
| Dodecylpyridinium bromide | 0·0175 | 0·010 |
| *Dodecylpyridinium iodide | 0·0045 | 0·0043 |
| Tetradecylpyridinium chloride | 0·0140 | 0·0040 |
| *Tetradecylpyridinium bromide | 0·0028 | 0·00305 |
| *Cetylpyridinium chloride | 0·0009 | 0·00085 |
| *Cetylpyridinium chloride in 0·007M-sodium chloride solution | 0·00025 | 0·00020 |
| *Cetyltrimethylammonium chloride | 0·0010 | 0·00096 |

## Conclusions

Although the interfacial activity of an aqueous solution is only one of the factors that influence the stability of dilute emulsions or suspensions, some of the processes described above, which are closely associated with interfacial activity, have a direct bearing on such stability when fleece is present. The results described in this paper show that these processes are influenced considerably by change in chain length and halide ion of the wetting agent. As the chain length is decreased in the $C_{18}$ to $C_{12}$ range, it is possible to maintain a higher concentration of wetting agent in solution; the long-chain cation–anion complex becomes more soluble, and, with the chlorides, flocculation of suspended material, which is considerable at $C_{16}$, does not occur at shorter chain lengths. Some of the difficulties associated with the use of cationic wetting agents of the longer chain lengths may therefore be overcome by the use of tetradecyl and dodecyl compounds, provided that a suitable halide ion is selected.

## Acknowledgments

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The University, Nottingham

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# THE NON-VOLATILE ORGANIC ACIDS OF GRASS

By A. C. HULME and A. RICHARDSON

The non-volatile organic acids are extracted from grass and separated from amino-acids, sugars, etc., by ion-exchange chromatography. The acid-containing fractions displaced from the anion-exchange columns are further separated by partition chromatography on buffered silica gel.

One of the acids present is proved to be quinic acid. By means of filter-paper chromatography, the presence of succinic, malic, malonic, and citric acids is indicated, thus confirming earlier work. Chlorogenic acid, not previously reported in grass, was also shown to be present. At least two other acids, whose identities are not established, are present, one of which appears to be allied constitutionally to quinic acid.

By the use of classical methods of isolation and characterization, Nelson & Hasselbring[1] and Nelson & Mottern[2] showed the presence of aconitic, citric, malic, malonic, oxalic, and tricarballyic acids in extracts of various graminaceous plants. More recently, Davies & Hughes,[3] employing the modern techniques of ion-exchange and filter-paper chromatography, have shown the presence of some of these acids, as well as succinic acid and several ' unknown ' acids, in extracts of forage grasses.

As a preliminary to the identification of these unknown acids, a survey was made of 80%-alcohol extracts of mixed herbage grass. The presence of citric, malic, malonic, and succinic acid was confirmed. In addition, the presence of quinic acid was unequivocally established; another acid that has properties similar to quinic acid, but the identity of which is not yet certain, was detected, and further chromatographic results obtained for the other ' unknown ' acids present. Chlorogenic acid was also found to be present.

## Experimental

The material used was mixed herbage consisting mainly of meadow foxtail and meadow fescue, which had been dried in a commercial grass-drier immediately after cutting, ground to a fine powder in a Christy & Norris mill, and well mixed.

This powder (100 g.) was extracted with 80–85% ethanol in a vacuum extractor[4] until the extracting liquid remained colourless. The alcohol was evaporated off under reduced pressure at 45° and the resulting green opalescent liquid filtered through a pad of asbestos; the pad was washed with hot water. The filtrate and washings were combined and shaken overnight with sufficient deactivated charcoal[5, 6] to remove all the colouring matter. The charcoal was filtered off and washed until free of acid. The filtrate and washings were made up to 500 ml. and found to contain 580 mg. of acid, expressed as malic acid. The colourless liquid was passed down a column of Zeo-Karb 215, large enough to remove all amino-acids and cations. The effluent and washings from the Zeo-Karb column (1000 ml., containing 1·83 g. of acid as malic acid; this indicates the large amount of the acids originally present as salts) were passed down a column containing 20 g. (70–100 mesh) of Amberlite IR 4B, on which the acids were absorbed. A strongly basic ion-exchange resin must not be used for this primary absorption of the organic acids since lactic and other organic acids are formed by the action of the resin on the sugars present.[7, 8] For the same reason the Amberlite column was washed with water until tests showed the water leaving the column to be free from sugars. The acids were displaced from this column with 0·1N-hydrochloric acid, continuing until the effluent gave a faintly positive test for chloride. It has been found generally that, when displacing organic acids from anion-exchange resins, small amounts of citric and malonic acids (which have relatively high p$K$ values) still remain on the column when chloride ' breaks through ', and can be removed completely only by continuing the displacement with hydrochloric acid for a longer period. Paper chromatographic tests showed that, in the present instance, the proportion of these acids lost by stopping displacement of the acids for subsequent fractionation at a stage when a faintly positive test for chloride was given was very small. The Amberlite column was washed with water and the combined displacement liquid and washings (about 1 litre) were, after removal of carbon dioxide, passed down a Dowex 2 column (25 g. of resin of 100–150 mesh) at a rate of 6 ml. per hour, taking precautions to prevent the entry of carbon dioxide into the system.[9] After all the solution had passed down the column, the column was washed with water free from carbon dioxide. The acids were then displaced by 0·1N-hydrochloric acid and the effluent flowing from the column at a rate of 6 ml. per hour was collected in 2-ml. fractions by means of a mechanical fraction-collector. Fraction 49 gave the first faintly positive test for chloride; 52 fractions were collected in all,

and each fraction was examined by paper chromatography. Standard paper-chromatographic techniques were used throughout, but details of the actual solvent systems used and the conditions under which chromatograms are run at Ditton Laboratory have been described elsewhere.[9] In addition to the solvent systems described in that paper, the benzyl alcohol–*tert.*-butanol–*iso*propanol–formic acid–water system (' B.B.P.F.'), used by Stark, Goodban & Owens,[10] was also employed in the present work. The bromocresol green spray described by Phillips & Pollard[7] was found to be the best of several indicator sprays tried for revealing the position of the acid spots.

On the results of the chromatographic analysis of the fractions displaced from the Dowex 2 column, fractions were combined, evaporated to a small volume under reduced pressure at 45° and re-chromatographed. The acids present—on the basis of paper-chromatographic examination with appropriate marker-spots of pure acids for the 'known' grass-acids—in the combined fractions were as shown below. The ' unknown ' acids were given letters of the alphabet and will be discussed below.

Fractions 1–27 : Quinic acid, and acid B
Fractions 28–36 : Quinic acid, B, A, and succinic acid
Fractions 37–41 : A trace of quinic acid, a spot having a very low $R_F$ value and giving a positive test for phosphorus, malic acid, A, and succinic acid
Fractions 42–47 : A trace of quinic acid, malic acid. This combined fraction was discarded
Fractions 48–52 : A trace of an acid with a very high $R_F$ value (this acid was present in such small amount that it was not examined further here), malic acid, citric acid, malonic acid, hydrochloric acid

*Further fractionation of the combined fractions from Dowex 2 ; the use of silica-gel columns*

Various adaptations of separation of organic acids by means of partition chromatography have been reported since the original paper of Isherwood.[11] The modification developed by Bradfield,[12] which employs buffered silica-gel columns to shift the partition coefficients of a mixture of acids (the partition coefficient is dependent on the degree of dissociation of an acid and hence on the pH of the surrounding medium) in the direction of maximum difference, was used here. To the requisite amount of silica gel, prepared as described by Bradfield, two-thirds of its weight of phosphate buffer (a buffer to give a pH 2·9 was used throughout the present work) was added, and the mixture was well mixed by means of a glass rod with a flattened end. The resultant powder was made into a slurry with buffer-saturated methyl *iso*butyl ketone (the solvent advocated by Bradfield), and poured into a suitable glass column, which was already three-quarters full of the buffered solvent. The buffered solvent was prepared by shaking, in a separating funnel, redistilled methyl *iso*butyl ketone (MIK) with about 1/20 of its volume of phosphate buffer, allowing the lower aqueous layer to settle out, running this off and filtering the MIK through a filter paper to remove excess of water droplets. As the slurry of gel was run into the column the tap of the column was opened and solvent allowed to run through freely. When all the gel had packed into the column it was washed with buffered solvent for several hours at a rate of flow of about 60 ml. per hour. Before applications of the solutions of acids to be separated the solvent was allowed to fall just to the level of the silica gel. (When these silica-gel columns are used the phosphoric acid in the buffer does not leave the column, and so contaminate any organic acids undergoing separation, until solvent many times the volume of the silica gel has passed through.)

*Fraction* (1–27).—This fraction contained 487 mg. of acid calculated as quinic acid. Attempts were made to separate quinic acid and B on a Dowex 2 column (a 10-g. column of Dowex 2, 100–150 mesh) by means of 35% aqueous dioxan as ' solvent ', according to the method of Owen.[13] This use of a miscible mixture of water and organic solvent is aimed at a combined use of ion-exchange and absorption chromatography. The acids were displaced from the column by 0·1N-hydrochloric acid in 35% dioxan. Although quinic acid tended to leave the column, on displacement, before acid B there was so much overlap that the method was abandoned, and the effluent was bulked and evaporated to dryness under reduced pressure at room temperature. The dry residue was dissolved in a minimum of *tert.*-pentyl alcohol[11] and carefully applied to the top of a buffered silica-gel column prepared as already described. When the acid solution had sunk into the gel, buffered MIK was carefully added from a reservoir attached to the top of the column (all joints were of glass). The effluent from the column was collected in fractions of about 10 ml., using a mechanical fraction-collector. Paper chromatographic tests showed that complete separation of the two acids was achieved. The fractions containing each acid were combined, evaporated to dryness under reduced pressure, dissolved in water and re-evaporated several times to remove all the MIK.

The identity of the quinic acid was confirmed by its m.p. (162–164°, corrected), alone and when admixed with an authentic sample of the acid. The *p*-bromophenacyl derivative was prepared and its melting point found to be 127°; the *p*-bromophenacyl derivative of authentic quinic acid melts at 125–127° (Hulme[9]). The sample, after oxidation with hydrogen peroxide, gave a positive reaction in the citrazinic acid test.[9] Sufficient of acid B was not obtained for its identification, but it also gave, on oxidation with hydrogen peroxide, a positive reaction in the citrazinic acid test. This would suggest that it has a structure similar to that of quinic acid; paper-chromatographic comparison with shikimic acid (quinic acid minus one molecule of water, see below) showed that the two acids were not identical.

The paper-chromatographic evidence for the identity of quinic acid from grass, and the comparison of B with shikimic acid, are shown in Fig. 1. Acid B and shikimic acid have identical $R_F$ values in butanol–formic acid–water [solvent (1)] but not in B.B.P.F. [solvent (2)] and propanol–ammonia solution–water [solvent (3)], although the $R_F$ values of the two acids in the last two solvents are very close to one another.

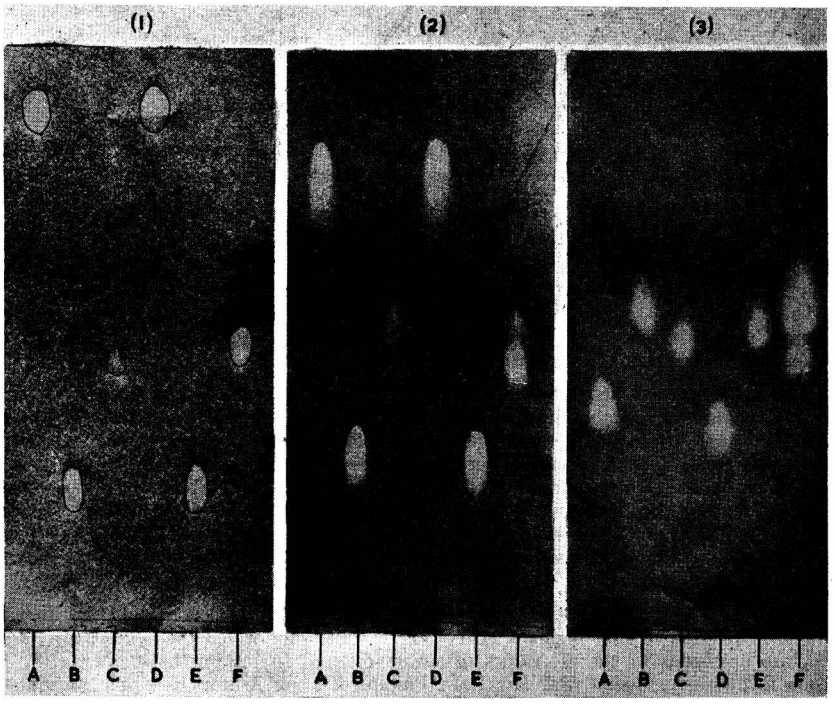



FIG. 1.—*Chromatograms in three solvent systems showing the identity of malonic acid and quinic acid from grass and the non-identity of acid B with shikimic acid*

A Authentic malonic acid
B Authentic quinic acid
C Authentic shikimic acid
D Malonic acid from grass + authentic malonic acid
E Quinic acid from grass + authentic quinic acid
F Acid B from grass + authentic shikimic acid

Solvent systems
(1) Butanol–formic acid–water
(2) Benzyl alcohol–*tert.*-butanol–*iso*propanol–formic acid–water
(3) Propanol–ammonia solution–water

*Fraction* (28–36).—This combined fraction was not examined further since the amount of acid A present was very small.

*Fraction* (37–41).—This fraction was evaporated to dryness *in vacuo* and dissolved in 5 ml. of MIK. It was run on to a column of 6 g. of silica gel buffered to pH 2·9, buffered MIK, was run through and the effluent collected in fractions of 5–6 ml.; 50 such fractions were collected. Paper chromatograms showed that fractions 1–10 contained succinic and

malic acids (confirmed by equilibrated paper chromatograms run in three solvents with marker-spots and mixed spots using authentic samples of the two acids); fractions 11–18 contained acid A only; fractions 19–30 contained acid A and a spot having a low $R_F$ value in acid solvents, which gave a positive test for phosphate (therefore, probably, phosphoric acid); fractions 31–50, when combined and evaporated to a small volume, showed traces only of quinic acid. There was insufficient of acid A in fractions 11–18 for its identification; its $R_F$ values in various solvents are given in Table I. It is interesting that all the other acids present, as well as such acids as aconitic, tricarballyic and tartaric in 'synthetic' mixtures, leave silica-gel columns in MIK in the same order as they travel on filter paper with the butanol–formic acid–water solvent system, whereas acid A travels faster on paper in this solvent than malic acid although, as mentioned above, it moves more slowly down the silica-gel column than malic acid. This suggests that it may have a constitution other than that of a simple carboxylic or hydroxy-acid. Paper chromatographic examination of the product of the interaction of the acid with 2 : 4-dinitrophenylhydrazine suggested that it may be a keto-acid.

**Table I**

| | Solvent | | | | | |
|---|---|---|---|---|---|---|
| | A | | B | | C | |
| Acid | $R_F$ | $R_{MA}$ | $R_F$ | $R_{MA}$ | $R_F$ | $R_{MA}$ |
| A | 0·49 | 1·08 | 0·61 | 1·36 | 0·50 | 1·63 |
| B | 0·31 | 0·68 | 0·33 | 0·75 | 0·45 | 1·46 |
| Shikimic | 0·31 | 0·68 | 0·37 | 0·84 | 0·38 | 1·22 |

*Fraction* (48–52).—This fraction was evaporated to dryness, dissolved in 35 ml. of *tert.*-pentyl alcohol (as a whole it was not very soluble in MIK) and fractionated on a buffered silica-gel column (18 g. of gel), with buffered MIK as solvent.

Three acids were separated and identified by paper-chromatographic comparisons with authentic specimens (see Fig. 1) as malonic, malic, and citric acids. Citric acid was confirmed by means of the citrazinic acid test.[14]

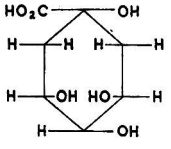
*Chlorogenic acid.*—A separate water-extract of 10 g. of the dried grass sample was made, filtered through asbestos, concentrated and extracted with ethyl acetate. The ethyl acetate extract was concentrated to a small bulk and paper chromatograms were run in butanol–acetic acid–water. A spot appeared which was blue in ultra-violet light and turned to duck-egg green on exposure to the fumes of ammonia. Comparison with a spot of chlorogenic acid run alongside, and also mixed with the ethyl acetate extract, and treated with the spraying reagents listed by Hulme,[15] provided strong evidence that this compound in the grass extracts was chlorogenic acid. Attempts are being made to isolate chlorogenic acid in quantity from grass.

$R_{MA}$ *values.*—In Table I not only are the $R_F$ values of the acids given, but also their rate of travel in relation to that of malic acid. These values, for which the symbol $R_{MA}$ is proposed, are analogous to the $R_G$ values of sugars referred to tetramethyl-D-glucose.[16] This decision follows a discussion with Mr. J. D. Phillips of Long Ashton Research Station, who has been, independently, using the same convention in his work on the acids of fruit juices. It has been found over a long period in this Laboratory that the $R_F$ value of malic acid is more constant and reliable than that of many acids. Malic acid occurs in most plants and is also readily obtained in the pure state.

## Discussion

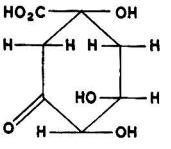
In confirmation of earlier work and the more recent work of Davies & Hughes,[3] the presence of succinic, malic, malonic, and citric acids in grass has been detected. In addition, quinic acid has been shown for the first time to be present in grass. Evidence is accumulating of the widespread occurrence of this acid in the plant world. Once again its presence is associated with that of chlorogenic acid.[15] From the position of the acids on displacement from the Dowex 2 columns, their $R_F$ values in basic solvent systems, and the suggestion by Davies & Hughes that the two acids concerned were monobasic acids of relatively high molecular weight, it appears that quinic acid may be acid F of Davies & Hughes and acid B may be identical with Davies & Hughes' acid G. It is difficult to see where acid A of the present paper fits in

to the results of Davies & Hughes. As already mentioned, there is some evidence for regarding acid B as similar in constitution to quinic acid. It seems possible that this acid may turn out to be 5-dehydroquinic acid, which Davis[17] has pointed out may be an intermediate in the metabolism of quinic acid by *Aerobacter* spp.



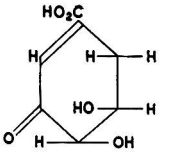
(I)

Quinic acid



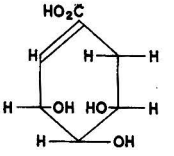
(II)

Dehydroquinic acid



(III)

Dehydroshikimic acid



(IV)

Shikimic acid

All four of these compounds, (I), (II), (III) and (IV), would, on oxidation, break down to citric acid or aconitic acid and so give a positive reaction in the citrazinic acid test.

The presence of malonic acid in grass is interesting in connexion with the possible operation of the Krebs citric acid cycle in plants,[18] for by inhibiting the action of succinic dehydrogenase this acid will prevent the operation of the full sequence of operations of the citric acid cycle.

**Acknowledgments**

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# CONNECTIVE TISSUE OF MEAT. III.*—Determination of Collagen in Tendon Tissue by the Hydroxyproline Method

By L. C. BAKER, L. H. LAMPITT and K. P. BROWN

Samples of ' collagen ' have been prepared from tendon dissected from shin of beef and found to have a maximum hydroxyproline content of 13·2%, giving a factor of 7·57 for converting hydroxyproline into ' collagen '. A comparison has been made of the results obtained for the ' collagen ' content of freeze-dried tendon preparations (*a*) by the hydroxyproline method and (*b*) by the modification of the Lowry, Gilligan & Katersky method previously published.

Collagen fibres were originally identified by the histologist on the basis of their structure and staining reactions ; they were found to be the major components of many connective tissues. These fibres were later found to possess certain chemical properties, notably (i) dissolution and breakdown to gelatin on being heated with water, (ii) stability to certain enzymes, e.g. alkaline trypsin, which attack other proteins, and (iii) stability to certain chemical reagents, dilute acids and alkalis and salt solutions, which dissolve other proteins. More recently, the investigation of the structure of collagen fibres with the electron microscope and by *X*-ray-diffraction methods has revealed a common crystal lattice for collagen fibres from many sources but, as Bear[1] has pointed out, such methods cannot establish chemical identity : ' —the situation is analogous to the phenomenon of isomorphism encountered in simple substances, wherein chemically similar but not identical species may replace one another in almost identical crystal lattices. In the collagens amino-acid residues may be presumed to be replaceable units within the fibrils which retain a basic type of organisation.'

An initial difficulty in the establishment of collagen as a chemical entity is the insolubility of collagen fibres, from many sources, in water and in mild chemical reagents, but the recent work of Neuman & Logan,[2] in which they found that ' collagen ' prepared from various materials was unique among proteins in having a high and fairly constant hydroxyproline content, indicates one line of attack on this problem.

The preparation of samples of ' pure collagen ' has been described by a number of authors : (1) Highberger[3] extracted the corium of cattle hide with alkaline trypsin ; the residue was washed, dried and ground to a powder. The powder was extracted with alcohol, freed from solvent and then extracted with half-saturated lime-water. Finally, the residue was washed successively with very dilute acetic acid and water, dehydrated with alcohol and ether and air-dried. (2) Bergmann & Stein[4] extracted Achilles tendon of cattle, comminuted by mincing it with solid $CO_2$, with 10% sodium chloride solution followed by M/15-disodium hydrogen phosphate solution. The residue was kept at 0° under toluene. (3) Bowes & Kenten[5] restricted the preparation of ' collagen ' from hide to extraction with 10% sodium chloride solution and removal of the grain layer and a thin layer on the flesh side, followed by degreasing. They preferred to accept the presence of small amounts of elastic and reticular tissue rather than risk possible modification of the collagen by treatment with alkaline and enzymic reagents. (4) Neuman[6] prepared samples of ' collagen ' from cattle hide and bone, the Achilles tendon of cattle, pig and sheep, the tarso-metatarsal tendon of chicken and the tail tendon of cattle, rat and kangaroo. Apart from one preparation by Highberger's method, the materials, after comminution in a Waring Blendor, were extracted with 10% sodium chloride solution, followed by disodium hydrogen phosphate ; the residues were dehydrated, defatted and air-dried.

**Table I**

*Analysis of tendon samples*

| | Beef tendon | | | | Veal tendon | |
|---|---|---|---|---|---|---|
| | I | II | III | IV | I | II |
| Total solids of comminuted fresh tendon, % | 41·0 | 35·2 | | | 29·2 | 29·9 |
| Total solids of freeze-dried tendon powder, % | 89·3 | 87·8 | | | 90·6 | 86·8 |
| Fat content of freeze-dried tendon powder, % | 1·2 | | 5 | 15·3 | 4·75 | |

In the work reported here, samples of ' collagen ' have been prepared from tendon dissected from shin of beef by the method of Bowes & Kenten,[5] and also by a method to be described in the Experimental section. These samples were used for the determination of hydroxyproline, and thus to establish a factor for converting hydroxyproline into collagen. This factor having

* Part II : *J. Sci. Fd Agric.*, 1953, **4**, 165

been established, the estimation of hydroxyproline was used to determine collagen in preparations of tendon dissected from shin of beef, for comparison with results obtained by other methods and, in particular, the modification of the Lowry, Gilligan & Katersky method published by the present authors.[7]

## Experimental

(1) *Preparation of tendon*

The tendon was dissected from shin of beef on four occasions and from shin of veal on two occasions; it was converted into freeze-dried, defatted powder by the method already described.[7] Analytical results obtained on these samples are recorded in Table I.

(2) *Preparation of 'collagen' samples from defatted, freeze-dried tendon powder*

*Method A.*—The defatted freeze-dried beef-tendon powder (4 g.) was treated in the Blendor for 60 seconds with 0·1N-sodium hydroxide solution (150 ml.) to dissolve cellular material and any adhering muscle protein. The suspension was centrifuged and the insoluble matter washed once with 0·1N-sodium hydroxide solution. The swollen material obtained on centrifuging was again suspended in water, neutralized carefully with dilute hydrochloric acid, centrifuged and the residue washed once with water. The residue from the water-washing was then treated in the Blendor with 0·01N-hydrochloric acid for 5 minutes, using a total of 2000 ml. of the acid; a very viscous solution was obtained which contained only a little suspended matter. The acid suspension was centrifuged and the supernatant liquid was then carefully neutralized with 0·1N-sodium hydroxide solution with constant stirring. Thick fibrous clots of precipitated collagen formed which were washed with water after centrifuging. The fibrous residue was suspended in alcohol for several hours, centrifuged and the residue suspended overnight in ether. The residue from the ether extraction, after air-drying at 30°, still retained some alcohol. After treatment in the freeze-drier a tough fibrous mass was obtained which was passed through the Raymond mill with the $\frac{1}{16}$-in. sieve.

*Method B.*—The method of Bowes & Kenten,[5] for the preparation of collagen from ox hide, was adapted to defatted, freeze-dried beef-tendon powder as follows: 2 g. of powder was treated in the Blendor for 30 seconds with 10% sodium chloride solution. The suspension was centrifuged and the slightly cloudy supernatant extract was discarded. The residue was suspended in fresh 10% sodium chloride solution and set aside at 35° F for about 64 hours. The suspension was centrifuged and the residue washed once with water and dehydrated with acetone. On air-drying, a tough fibrous mass was obtained, which was freeze-dried and powdered. The analytical results obtained on the samples of prepared collagen are recorded in Table II.

**Table II**

*Analysis of collagen samples*

| Method | A | A | A | B | B |
|---|---|---|---|---|---|
| Sample | I | II | III | IV | V* |
| Total solids, % | 85·6 | 89·2 | 88·3 | 85·2 | 89·5 |
| Total nitrogen, % | 15·4 | | 15·7 | 14·7 | 15·6 |
| Total nitrogen (as per cent. of solids) | 18·0 | | 17·8 | 17·2 | 17·4 |

* Prepared from freshly dissected (not freeze-dried) tendon

(3) *Determination of hydroxyproline in collagen samples*

Except for sample I, hydroxyproline was determined in the collagen samples (*a*) after direct acid hydrolysis and (*b*) after preliminary autoclaving with water, followed by hydrolysis of an aliquot of the aqueous autoclave extract with acid; the hydroxyproline of sample I was determined only by method (*b*).

(*a*) *Direct acid hydrolysis.*—Generally about 0·05 g. of collagen was accurately weighed and hydrolysed by heating with 2 ml. of 6N-hydrochloric acid for 6 hours at 25 lb. pressure in a sealed tube. After being cooled the tube was opened and the solution was neutralized and diluted to 200 ml.; of this solution, aliquots of 1 ml. were used for colour development (cf. Baker, Lampitt & Brown[8]).

In adapting the Neuman & Logan method,[2] the coloured solutions are prepared in test-tubes 6 in. × $\frac{5}{8}$ in. graduated at 10 ml.; at any one time the colour may be developed in three pairs of duplicate standards and three pairs of duplicate unknowns. The colour is developed as described by Neuman & Logan except that (i) the range of concentration of hydroxyproline is 15–40 μg. per 10 ml. of coloured solution, (ii) 0·05M-copper sulphate solution is used instead of

0·01M, (iii) special care is taken to ensure rapid and thorough mixing after the addition of each reagent and (iv) the colour density is measured as soon as possible after colour development is complete, taking the solutions in order of decreasing density (whether they are standards or unknowns) and using a 1-cm. cell.

(*b*) *Acid hydrolysis of aqueous autoclave extract.*—Approximately 0·1 g. of collagen was accurately weighed and autoclaved with 40 ml. of water for 3 hours at 15 lb. pressure; after being cooled and centrifuged the supernatant extract was decanted into a 100-ml. graduated flask. The residue was autoclaved with a further 30–40 ml. of water for 3 hours at 15 lb. pressure, the extract was decanted into the same flask, and diluted to the mark. Of the total extract, 10 ml. was made just acid and evaporated to dryness in a test-tube; 1 ml. of 6N-hydrochloric acid was added to the residue and, after being sealed, the tube was autoclaved at 25 lb. pressure for 6 hours. The hydrolysate was cooled, neutralized and diluted to 50 ml.; of this solution, aliquots of 1 ml. were used for colour development.

The results obtained are recorded in Table III.

**Table III**

*Hydroxyproline content of collagen samples, as per cent. of total solids*

| Sample No. | Collagen prepared by Method A | | | Collagen prepared by Method B | |
|---|---|---|---|---|---|
| | I | II | III | IV | V* |
| Direct acid hydrolysis | | 12·7 | 13·3 | 12·5 | 12·5 |
| | | 12·8 | 13·2 | | 12·2 |
| | | | | | 12·7 |
| | | | | | 13·0 |
| Hydrolysis of autoclave extract | 13·2 | 12·0 | 13·3 | 12·2 | 12·8 |
| | 13·0 | 12·1 | 13·3 | 11·5 | 12·6 |
| | 13·3 | | | 11·6 | |
| | 13·2 | | | 12·0 | |

* With freshly dissected tendon

The results in Table III show: (i) That the average hydroxyproline contents (13·2%) of collagen samples I and III prepared by Method A were higher than those (12·3%) of samples IV and V prepared by Method B. The higher figure, 13·2%, is in good agreement with figures given by Neuman[6] for tendon. If 13·2% of hydroxyproline is taken as the true figure for collagen prepared from tendon dissected from shin of beef, the factor for conversion to collagen is 7·57. (ii) That the results obtained by direct acid hydrolysis and by acid hydrolysis of the autoclave extract are in good agreement for collagen sample III, where the highest hydroxyproline content of the samples examined was found; the agreement for results by the two methods is less satisfactory where a somewhat lower hydroxyproline content was found, suggesting that these collagen samples were not pure.

(4) *The collagen content of defatted, freeze-dried tendon powder*

In this section the results obtained by the following two methods of determining collagen are compared: (i) by determination of hydroxyproline and (ii) by weight difference, before and after autoclaving with water, by use of the modification of the Lowry, Gilligan & Katersky method published by the present authors.[7] The effects of preliminary extractions of the tendon powder with certain reagents on the results obtained by the two methods have also been studied; the reagents used were (*a*) 0·3M-disodium hydrogen phosphate solution, pH 9·0; (*b*) citrate buffer at pH 3·75; and (*c*) 0·1N-sodium hydroxide solution.

For the determination of hydroxyproline in the hydrolysate obtained by direct acid hydrolysis, 0·05–0·10 g. of tendon powder was treated as described under (3*a*). For the remaining determinations 0·20–0·25 g. of tendon powder was dried and weighed before and after the aqueous autoclaving treatment described under (3*b*); the difference in weight was taken as 'collagen' for comparison with the result obtained by determining hydroxyproline in the acid hydrolysate of an aliquot of the aqueous autoclave extract. In many experiments the tendon powder was given a preliminary extraction treatment in the Blendor with 150 ml. of reagent for 30 sec., but only 50 ml. of reagent was used when the blending treatment was omitted (see Table IV). The residue was washed once with the reagent before being neutralized, then washed with water, alcohol and ether. The results are given in Table IV.

From the results for collagen obtained by the hydroxyproline method, it may be stated that practically the same result was obtained by direct acid hydrolysis of tendon (I) and by

## Table IV

*Collagen content of defatted, freeze-dried tendon preparations, as percentage of total solids*

| | Direct acid hydrolysis | Aqueous autoclave extract | | | | | | After extraction with 0·1N-NaOH | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | I | II | | III | | IV | | V | | VI | | VII | |
| Preliminary extraction | — | None | | After blending for 30 sec. with phosphate buffer | | After blending for 30 sec. with citrate buffer | | In blender for 30 sec. | | Adding NaOH after blending for 30 sec. in water | | Stirring | |
| Determination | H-P | H-P | Wt. diff. | H-P | Wt. diff. | H-P | Wt. diff. | H-P | Wt. diff. | H-P | Wt. diff. | H-P | Wt. diff. |
| Sample BT I | 88·5<br>85·7<br>88·1<br>89·7 | | | | | | | 71·0<br>68·8<br>75·4(*c*)<br>75·1(*c*) | 73·7<br>73·6 | 77·9 | 81·6<br>83·7 | 80·5<br>78·4 | 86·8<br>85·7 |
| Sample VT I | | | | | | | | 57·2<br>56·7 | 61·6<br>62·1 | | | 71·5<br>67·6 | 72·4<br>72·7 |
| Sample BT II | F2(*a*) 78·1<br>F2(*a*) 76·5<br>F4(*b*) 75·8<br>F4(*b*) 75·6 | 77·5<br>78·3 | 93·0<br>93·4 | | | | | | | | | | 74·9<br>75·6 |
| Sample VT II | F2(*a*) 74·7<br>F2(*a*) 72·5<br>F4(*b*) 60·7<br>F4(*b*) 62·9 | 73·0<br>73·0 | 92·0<br>91·2 | | | | | | | | | | 69·6<br>69·1 |
| Sample BT III | | | | 77·5<br>76·6 | 84·3<br>83·2 | 75·7 | 87·3 | 76·1<br>73·3 | 77·3<br>74·8 | | | | |
| Sample BT IV | | | | | | 76·0<br>76·4 | 87·3<br>89·4 | 72·3<br>71·9 | 73·5<br>73·6 | | | | |
| | 82·0<br>81·5 | 80·7<br>80·7<br>80·9 | 93·4<br>93·2<br>93·0 | | | | | 72·1<br>72·1<br>72·9 | 76·5<br>76·6<br>77·5 | | | | |

H-P Hydroxyproline
(*a*) Fraction passing 32 mesh, retained by 48 mesh
(*b*) ,, ,, 60 mesh
(*c*) Residue after extraction with sodium hydroxide solution, not dried but hydrolysed at once

acid hydrolysis of an aliquot of the extract from the direct aqueous autoclaving (II), e.g. (average figures) for sample BT II F 2: 77·3% compared with 77·9%; for sample VT II F 2: 73·6% compared with 73%; and for sample BT IV: 81·7% compared with 80·8%. By the method of direct acid hydrolysis (I) the hydroxyproline in any elastin present (1·8% according to Neuman[6]) would be counted as collagen, but the difference between the hydroxyproline contents of collagen and elastin would require 7% of elastin to increase the apparent collagen content by 1%. The amount of collagen found by acid hydrolysis of an aliquot of the aqueous extract of the residue left after extraction of tendon powder with 0·1N-sodium hydroxide solution (V) is always less than that found by methods (I) and (II), e.g. for sample BT I: 72·6% compared with 88·0%; and for sample BT IV: 72·3% compared with 81·7% and 80·8%. This result suggests that a little collagen is dissolved when tendon powder is treated in the Blendor for 30 seconds with 0·1N-sodium hydroxide solution; cf. Lampitt, Baker & Brown.[7] This is demonstrated to be so in the next section. The results obtained for tendon powder BT I indicate that the less violent the treatment of the tendon powder in the Blendor, the less the collagen dissolved: 79·5% collagen found when the powder is stirred with 0·1N-sodium hydroxide solution (VII), 77·9% when the powder is blended with water and sodium hydroxide is added to give a concentration of 0·1N (VI) and 72·6% when the powder is blended with 0·1N-sodium hydroxide solution (V).

If the results obtained for collagen by the weight-difference method are compared with those obtained by the hydroxyproline method, autoclaving directly with water (II) clearly extracts some material that is not collagen, for sample BT II F 2: 93·2% compared with 77·9% (by hydroxyproline); for sample VT II F 2: 91·6% compared with 73%; and for sample BT IV: 93·2% compared with 80·8%. After preliminary extraction with 0·1N-sodium hydroxide solution to remove muscle and cellular material (V), the discrepancy is less marked but it must be remembered that a little collagen has been dissolved: for sample BT I: 73·6% compared with 72·3%; for sample VT I: 61·8% compared with 57·0%; for sample BT III: 76·0% compared with 74·7%; and for sample BT IV: 75·5% compared with 72·3%.

It is concluded from these results, particularly those for sample BT IV, that the weight-difference method, after removal of material made soluble by blending with 0·1N-sodium hydroxide solution (V), gives results for collagen that are slightly low, 75·5% compared with the true collagen content of 80·8% as determined by the hydroxyproline method in (II); the non-collagenous material, not dissolved in 0·1N-sodium hydroxide solution but made soluble when autoclaved with water, does not entirely compensate for the collagen dissolved by the blending treatment with 0·1N-sodium hydroxide solution.

(5) *The collagen content of the material extracted from tendon powder by blending with* 0·1N-*sodium hydroxide solution*

In order to estimate the amount of collagen dissolved, 0·5 g. of sample BT I was treated in the Blendor for 30 seconds with 150 ml. of 0·1N-sodium hydroxide solution; the suspension was centrifuged and the clear extract was filtered through glass wool into a 200-ml. graduated flask; the residue was washed once with 0·1N-sodium hydroxide solution, and the washings were filtered into the same flask and the solution therein diluted to the mark. Four aliquots of 10 ml. of this solution, after each had been acidified with approximately 0·5 ml. of 2·5N-hydrochloric acid, were evaporated to dryness successively in the same test-tube and the total residue was hydrolysed with 2 ml. of 6N-hydrochloric acid for 6 hours at 25 lb. pressure in a sealed tube. The hydrolysate was neutralized and diluted to 25 ml.; 1 ml. of this solution was used for colour development. Duplicate acid hydrolyses were carried out and the collagen equivalent to the average amount of hydroxyproline found amounted to 6·2% of the total solids in sample BT I.

## Summary

(1) Samples of 'collagen' were prepared from tendon dissected from shin of beef by the use of (*a*) a method involving dissolution of collagen in dilute hydrochloric acid and reprecipitation on neutralization and (*b*) the method of Bowes & Kenten.

(2) Two of the three samples of 'collagen' prepared by method (*a*) were found to contain 13·0–13·3% of hydroxyproline; the remaining samples contained 11·5–12·8% of hydroxyproline. If 13·2% is taken as the true hydroxyproline content of 'collagen' from shin of beef, the factor for converting hydroxyproline to 'collagen' is 7·57.

(3) A comparison was made of the collagen content of freeze-dried tendon preparations (*a*) by the hydroxyproline method and (*b*) by the modified Lowry, Gilligan & Katersky method. Estimation of hydroxyproline in (i) a direct acid hydrolysate, (ii) an acid hydrolysate of an

aqueous autoclave extract and (iii) a hydrolysate prepared as in (ii), but after an initial extraction with 0·1N-sodium hydroxide solution, showed that similar results were obtained in (i) and (ii), whereas (iii) gave slightly lower results, indicating that a little collagen was dissolved in the initial extraction with 0·1N-sodium hydroxide solution; this was confirmed by analysis. The collagen determined by loss of weight on autoclaving (iii) was slightly higher than that found by hydroxyproline in (iii) but less than that found by hydroxyproline in (i) and (ii); this indicates that the collagen lost by dissolution in 0·1N-sodium hydroxide solution in (iii) is not quite compensated by the non-collagenous material left undissolved by the 0·1N-sodium hydroxide solution, but which is soluble on being autoclaved with water.

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# A $^{14}C$ STUDY OF CARBON DIOXIDE FIXATION IN THE APPLE. I.—The Distribution of Incorporated $^{14}C$ in the Detached McIntosh Apple*

By N. ALLENTOFF, W. R. PHILLIPS and F. B. JOHNSTON

Mature, stored McIntosh apples exposed for 18 hours in darkness to $^{14}CO_2$ were found to incorporate $^{14}C$ in the malic acid, and in the aspartic and glutamic acids, α-alanine and serine of the nitrogenous fractions, indicating the operation of a system of the 'malic enzyme' type.

## Introduction

In 1928 Haynes & Archbold,[1] from an analysis of the curves of acid and carbohydrate loss, postulated the continuous production of malic acid in the stored apple, concurrently with its consumption. More recently, Thomas[2, 3] was led to conclude from studies of the acid metabolism of the Crassulaceae that the fixation of carbon dioxide in a reaction of the Wood & Werkman or 'malic enzyme' type was an important means of production of plant acids.

Thomas also mentioned the desirability of a study of this system in fruits that accumulate organic acids, by the use of carbon isotopes. In connexion with investigations of the respiration of apples in storage, one of us (W. R. P.) discussed this matter further with Professor Thomas, and, as a result, this study of the fixation of carbon dioxide in the mature, detached McIntosh apple was undertaken.

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Contribution No. 811 from the Horticulture Division, Experimental Farms Service, Canada Department of Agriculture

## Experimental

*Source of apples.*—The McIntosh apples used in these determinations came from a single tree in the orchard of the Central Experimental Farm, Ottawa. They were harvested on 1 October, 1952, and stored at 0°.

*Exposure to $^{14}CO_2$.*—In January, 1953, 12 apples were removed from storage, allowed to come to room temperature for 24 hours, and placed in an 8-litre, darkened vacuum-desiccator. The pressure in the desiccator was reduced to 35 cm. for 10 minutes. Into a small flask connected to the desiccator was weighed 2·5 g. of barium carbonate, sufficient to supply 5% of carbon dioxide containing 2 mc of $^{14}C$ to the free atmosphere of the desiccator. The carbon dioxide was generated by the addition of an excess of dilute phosphoric acid to the carbonate and swept into the desiccator with air free from carbon dioxide, bringing the pressure in the desiccator up to that of the atmosphere.

After 18 hours the desiccator was flushed with air free from carbon dioxide; the $^{14}CO_2$ was collected by absorption in 0·2M-barium hydroxide solution. The apples were placed in a freezer at − 20° until required.

*Preparation of alcoholic extracts.*—Four apples were removed from the freezer and thawed. The stems and seeds were discarded, leaving 504 g. of material. This was blended for five minutes in a Waring Blendor with sufficient ethanol to give a 70% alcoholic extract. After being set aside overnight the solution was filtered. The residue was washed with 70% alcohol and dried, to yield 11·6 g. of alcohol-insoluble matter. The filtrate was evaporated to dryness at 75° *in vacuo* to give 55·1 g. of alcohol-soluble solids, which was made up to 250 ml. with water (solution I).

*Determination of malic acid radioactivity.*—Paper chromatography, by the method of Lugg & Overell,[4] showed no acid other than malic to be present in comparable quantity. The acid was determined,[5] therefore, by titration of 10 ml. of solution I to pH 8·1 with 0·1N-sodium hydroxide solution, giving a concentration of 0·404% on the basis of fresh-apple weight.

A further 50 ml. of solution I was acidified with sulphuric acid and extracted continuously with ether for 72 hours. The malic acid was isolated from the extract for radio-assay as the aniline salt, the preparation of which is described in Part II of this paper.

*Examination of the sugars for radioactivity.*—The apples contained 6·4% of reducing sugars (fructose and glucose) and 1·9% of non-reducing sugars, as determined by the method of Hassid.[6]

The glucosazone[7] prepared from a portion of solution I, representing fructose and glucose, showed no measurable activity. On hydrolysis of a second portion of the solution with hydrochloric acid, the glucosazone obtained, now representing sucrose as well, showed a low activity, ascribed to the sucrose.

*The free amino-acids.*—The free amino-acids from 100 ml. of solution I were adsorbed on a column of Dowex 50 X-4 resin, 200–400 mesh, prepared by the same procedure as that used by Partridge[8] for Zeo-Karb 215. They were then eluted with 0·15N-ammonia solution to yield 0·171 g. (0·085% of fresh-apple weight).

A sample containing 0·7 mg. of this material was applied to a sheet of Whatman No. 4 filter paper in a spot 5 mm. in diameter, and resolved by ascending chromatography using 4 : 1 phenol–water in an ammoniacal atmosphere in the first dimension, and 4 : 1 : 1 *n*-butanol–acetic acid–water in the second. The resulting chromatogram (unsprayed) was pressed against a sheet of Ansco Non-Screen *X*-ray film for one month. Comparison of the darkened areas of the autoradiogram with the spots appearing on the chromatogram when sprayed with ninhydrin showed activity in the aspartic acid, glutamic acid and α-alanine regions, as tentatively identified by comparison of their positions with the data of Hulme & Arthington.[9]

The activities of these three amino-acids and serine were examined further. To each of two sheets of Whatman No. 4 filter paper, 40 mg. (assaying 40,000 counts per minute) of the amino-acid fraction was applied in a 52-cm. band 1 cm. wide. These were developed with the phenol solvent. The four bands containing the desired amino-acids, located by spraying strips from the sides and centre of the sheets with ninhydrin, were eluted with water and assayed for radioactivity. The first two bands contained the aspartic and glutamic acids, and required no further separation. The fractions containing the serine and α-alanine were reapplied to separate sheets and chromatographed with the butanol solvent; the appropriate bands were eluted and assayed as before.

The identity of each of the amino-acids thus separated was checked by chromatography with 2 mg. of authentic material, by using each of the two solvent sets, followed by elution and assay of the resulting single bands to ensure that the radioactivity travelled with the

standards. Since these manipulations involved losses that could only be estimated, and the assay of single samples in most instances, the results given in Table II are subject to errors of the order of ± 10% (relative).

*The amino-acids of the protein hydrolysate.*—A portion (11·2 g.) of the insoluble residue from the initial extraction with 70% alcohol was hydrolysed by refluxing with 200 ml. of 20% hydrochloric acid for 24 hours, yielding 3·75 g. of crude hydrolysate and 4·1 g. of insoluble residue. Treatment of the hydrolysate with Dowex 50 resin, as for the free amino-acids, gave 0·8 g. (0·162% of fresh-apple weight) of protein amino-acid fraction.

An autoradiogram of a portion of this fraction showed radioactivity principally in the aspartic and glutamic acids and serine. These three and the α-alanine were isolated, identified and assayed as described in the previous section.

*Plating and counting.*—Pads of the crystalline or powdered materials to be assayed were prepared by the method of Migicovsky & Evans.[10] Samples of the total extract and the amino-acid fraction were counted in shallow circular pans containing a disc of lens tissue, much as described by Calvin *et al.*[11] All samples were counted in a gas-flow Geiger counter and corrected for self-absorption from empirical correction curves for the substances concerned.

## Results and discussion

The corrected total activities of the fractions investigated are listed in Table I. The malic acid and free and hydrolysate amino-acids contained among them over 80% of the $^{14}C$ incorporated in non-volatile compounds, with over half of the total in the malic acid itself. Although all the amino-acid fractions separated by the chromatographic procedures showed some activity, a fact not surprising in view of the relatively long exposure of the apples to the $^{14}CO_2$, the four amino-acids listed in Table II accounted for over 75% of the activity in both the free and the protein fractions. The shift in activity from the α-alanine of the former to the serine (and glutamic acid) of the latter is noteworthy.

**Table I**

*Distribution of incorporated $^{14}C$*

| Fraction | Activity, % of total |
|---|---|
| Soluble in 70% alcohol | 87·1 |
| Malic acid | 63·8 |
| Sucrose | 1·6 |
| Free amino-acids | 10·1 |
| Insoluble in 70% alcohol | 12·9 |
| Hydrolysate amino-acids | 8·3 |

**Table II**

*Activity of individual amino-acids*

| Amino-acid | Activity, % of fraction total | |
|---|---|---|
| | Free amino-acids | Hydrolysate amino-acids |
| Aspartic acid | 39 | 23 |
| Glutamic acid | 9 | 21 |
| Serine | 2 | 28 |
| α-Alanine | 26 | 6 |

The finding that fixed activity is largely confined to malic acid and compounds that can be simply derived from it by the operation of the tricarboxylic acid cycle and transamination systems, is in accord with the initial uptake of carbon dioxide in a reaction of the 'malic enzyme' type.

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# A $^{14}C$ STUDY OF CARBON DIOXIDE FIXATION IN THE APPLE. II.*—Rates of Carbon Dioxide Fixation in the Detached McIntosh Apple†

By N. ALLENTOFF, W. R. PHILLIPS and F. B. JOHNSTON

The McIntosh apple in storage was found to produce malic acid by fixation of carbon dioxide at a rate of about half of the net daily acid loss. The contribution of this reaction to the production of amino-acids and protein was also quantitatively significant. The fixation activity rose to a high level at the time of harvest and continued to be high throughout the storage period. The rate of uptake of carbon dioxide increased with rising concentration of carbon dioxide in the external atmosphere.

## Introduction

In Part I of this study, a fixation of carbon dioxide in the dark by the mature, detached McIntosh apple was demonstrated, leading to the incorporation of $^{14}C$ in the malic acid and free and protein amino-acids of the fruit.

In order to assess the quantitative significance of this reaction as related to the metabolic changes of these components during the storage of the apple, it was necessary to arrive at an estimate of the rate of uptake of carbon dioxide under actual storage conditions, and to follow the fluctuations of this activity during the pre- and post-harvest periods. In this paper are presented the results of investigations of these factors during the 1952–53 season.

## Experimental

The apples used were part of the lot described in Part I, harvested on 1 October, 1952, and stored at 0° in a store-room of the ordinary commercial type. In order to determine the rate of fixation of carbon dioxide under these conditions, the apparatus was set up in the store-room, and samples of apples were exposed to decreasing concentrations of $^{14}C$-labelled carbon dioxide added to the storage atmosphere already present. The rate of uptake of carbon dioxide in storage was determined by extrapolating to zero concentration of carbon dioxide the plot of fixation rate against concentration of carbon dioxide supplied.

Comparison of malic acid activities with those of whole-apple-pulp preparations after apples were exposed to $^{14}CO_2$ showed that the activity incorporated in malic acid was consistently 50–65% of the total taken up. The total malic acid activity is thus an approximate index of the total carbon dioxide fixed. Given the malic acid concentration and its specific activity, the total uptake of carbon dioxide can be calculated, provided that the specific activity of the carbon dioxide available to the centres of fixation of the apple is known.

The carbon dioxide initially supplied to the atmosphere of the vacuum desiccator used for the exposures was of known activity, but on being passed into the apple this was diluted

* Part I: preceding paper

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by the unlabelled carbon dioxide already present in the fruit and continuously produced in its respiration. It was necessary, therefore, to carry out a series of runs in which the relation between the specific activity of the carbon dioxide inside the apple and that of the carbon dioxide initially supplied to the desiccator atmosphere was determined. The mean specific activity of the internal carbon dioxide thus obtained was the basis for calculating the uptake of carbon dioxide represented by a given activity of the malic acid.

Finally, in order to determine the amount of malic acid produced upon incorporation of a given amount of carbon dioxide, it was necessary to determine the positions of the $^{14}C$ in the acid molecule, and thence the stoicheiometry of the fixation reaction.

*Determination of the rate of fixation in storage.*—During January, 1953, samples of four apples each were exposed for 18 hours at 0° to external concentrations of 5, 2·5, 1, 0·2, 0·05 and 0·02% of carbon dioxide (containing from 0·5 to 0·005 mc of $^{14}C$) added to the storage atmosphere present in the vacuum desiccator, in the manner described in Part I. After the exposures, the external carbon dioxide was removed by flushing with air free from carbon dioxide for 30 minutes. The apples were then placed in a plastic bag, which was immersed in a water bath at 70° for 30 minutes, after which the juice was expressed in a hand-operated press. The malic acid content of this extract was determined as in Part I, and the aniline malate was isolated for counting as described in a later section.

The results for estimating the mean activities of the internal carbon dioxide were obtained from three runs at each of 5, 1 and 0·05% of carbon dioxide added to the external atmosphere. At each concentration, exposures of 1, 6 and 18 hours were made, after each of which the total internal carbon dioxide of the apples was collected by the method of Claypool,[1] precipitated as barium carbonate, and its specific activity compared with that of the reagent initially supplied.

*Seasonal variation in the rate of fixation of carbon dioxide.*—Samples of four apples each were removed from the tree or from storage and kept in the laboratory for 24 hours. They were then exposed to an atmosphere of 5% of carbon dioxide, of about 0·25-mc activity, for 18 hours at room temperature (22°). At the end of this period the apples were heated at 70°, the juice was expressed, the malic acid content determined and the aniline salt isolated for counting. A total of 25 such runs was carried out from 3 September, 1952 (nearly one month before harvest), to 26 March, 1953, by which time the fruit had passed the end of its useful storage life.

Determinations of the mean activity of the internal carbon dioxide were made in terms of the activity of the carbon dioxide initially supplied to the external atmosphere at a concentration of carbon dioxide of 5% and at 22°, with exposures of 1, 6 and 18 hours.

*Isolation of malic acid as the aniline salt.*—From 100 ml. of the expressed apple juice, acidified to pH 1–2 with sulphuric acid, the malic acid was separated by a 72-hour extraction with ether in a liquid–liquid extractor. The extracts were dried with anhydrous sodium sulphate and evaporated to dryness to give a residue containing 0·2–1·0 g. of malic acid, depending on the age of the apples.

The extract was dissolved in 20 volumes of ethyl acetate and an excess (0·5–1·0 g.) of aniline was added. The salt was precipitated, either spontaneously or upon being scratched with the spatula, in yields of 50–80% on the basis of the juice titrations. The melting point of the crude product was 136–138°. One recrystallization from boiling ethyl acetate containing a little decolorizing charcoal gave a 70% recovery of white, crystalline material, m.p. 137–139° (Rambech[2] reports 138–139°).

Analysis (carried out by the Laboratory of Dr. R. Dietrich, Zürich) (Found: C, 52·80; H, 5·74; N, 6·13. Calc. for $C_{10}H_{13}O_5N$: C, 52·85; H, 5·77; N, 6·16%). The original reference ascribes to this salt the composition aniline : malic acid in a 1 : 2 molecular ratio, whereas the analytical figures accord with a composition of aniline : malic acid in a 1 : 1 molecular ratio. A sample of the salt (m.p. 142–143°) prepared from authentic DL-malic acid gave a mixed melting-point of 135–137° with the apple material, and an identical *X*-ray-diffraction pattern. Although further recrystallization improved the melting point of the once-recrystallized salt, there was no significant change in its radioactivity. This material was thus sufficiently pure for radio-assay.

*Isolation and degradation of malic acid.*—Samples of juice from several of the seasonal variation determinations, which had been stored with three volumes of alcohol under refrigeration, were freed of alcohol by vacuum distillation and ether-extracted to give 3·4 g. of a brown oil. A portion of this (2·3 g.) was dissolved in 50 ml. of water, titrated to pH 8·5 with 0·2M-barium hydroxide solution, and the barium malate was precipitated by the addition of three volumes of 95% ethanol. The malate was redissolved in 50 ml. of water, stirred with

decolorizing charcoal, filtered and again precipitated with alcohol, yielding 1·3 g. of barium malate. The barium was precipitated by adding the calculated amount of sulphuric acid to the salt dissolved in 100 ml. of water, and filtered off on Whatman No. 50 filter paper supporting a bed of talc.

The filtrate was evaporated to dryness at 70° *in vacuo* to give 0·39 g. of waxy white residue. This was dissolved in 4 ml. of acetone and was precipitated as a colourless oil by being poured into 6 volumes of dry light petroleum. The solvent was decanted and the oil crystallized after several days *in vacuo* over phosphorus pentoxide, yielding 0·28 g. of (−)-malic acid, m.p. 97–101°. An authentic sample gave the same melting point and mixed melting point with the apple product.

The $^{14}C$ positions in this material were located by means of a total decarboxylation, by the method of Benson & Bassham,[3] and an α-decarboxylation in sulphuric acid, as described by Utter.[4]

## Results and discussion

*The initial fixation reaction.*—The total decarboxylation of the malic acid showed nearly all (99%) of the activity to be in the carboxyl groups. The selective α-decarboxylation indicated equal carboxyl labelling (50 ± 3% in the α-carboxyl group). These results are in accord with the operation of a system of the 'malic enzyme' type in the initial fixation reaction, in the presence of fumarase.[5, 6]

$$\overset{*}{C}O_2 + CH_3{\cdot}CO{\cdot}CO_2H \rightleftharpoons HO_2\overset{*}{C}{\cdot}CH_2{\cdot}CH(OH){\cdot}CO_2H$$

$$\rightleftharpoons HO_2\overset{*}{C}{\cdot}CH : CH{\cdot}\overset{*}{C}O_2H \rightleftharpoons HO_2\overset{*}{C}{\cdot}CH_2{\cdot}CH(OH){\cdot}\overset{*}{C}O_2H$$

Thus it is assumed, in the subsequent quantitative calculations, that one molecule of malic acid is formed for each molecule of carbon dioxide incorporated in this acid by fixation.

*The fixation rate in storage.*—From Figs. 1 and 2 the mean specific activities of the carbon dioxide of the internal atmospheres of apple were approximated by joining the plotted points with a smooth curve, and dividing the area under the curve by the total time (18 hours) of exposure, in each instance. The values obtained, expressed as percentages of the specific activities of the carbon dioxide initially supplied to the external atmospheres, are plotted in Fig. 3 against the corresponding concentrations of external carbon dioxide.

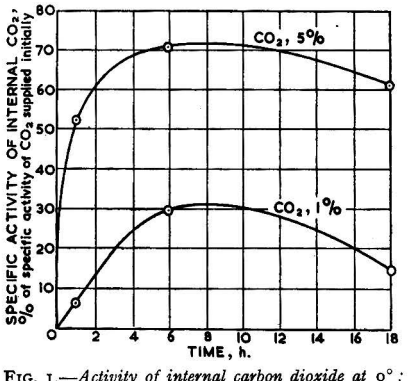


FIG. 1.—*Activity of internal carbon dioxide at 0°; 5% and 1% of carbon dioxide initially supplied to the external atmosphere*

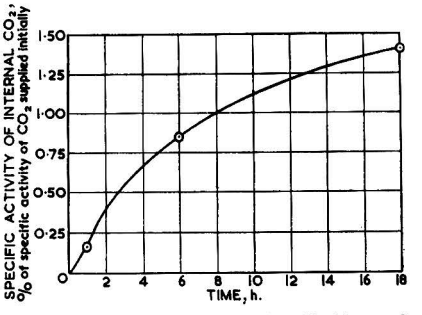


FIG. 2.—*Activity of internal carbon dioxide at 0°; 0·05% of carbon dioxide initially supplied to the external atmosphere*

By means of the total malic acid activity, the initial specific activity of the carbon dioxide supplied externally, and the ratio of internal to external activity as obtained for the appropriate concentrations of external carbon dioxide from Fig. 3, the rates of fixation were calculated for the determinations made at 0° for the range of initial external carbon dioxide concentrations (5% to 0·02%) used. These results are plotted in Fig. 4, expressed as mg. of malic acid produced by 100 g. of fresh weight of apple in 24 hours. The relation between rate of fixation and concentration of external carbon dioxide appears to be approximately linear, and the mean straight line, extrapolated to zero carbon dioxide added, gives a rate of production of malic acid of about 0·8 mg. daily per 100 g. of apple.

This value is about one-half of the net daily rate of consumption of malic acid at this period of storage. The activity incorporated in malic acid corresponds to about 0·25 mg. of carbon dioxide daily, and since this represents approximately half of the total uptake of carbon dioxide, the total is about 0·5 mg. per day. The respiratory production of carbon dioxide under the same conditions is about 10 mg. daily.

It was found in Part I that approximately 10% of the total activity taken up was incorporated in the free amino-acids and a similar amount in the protein. If it is assumed that one molecule of the weight of aspartic acid results eventually from the fixation of one molecule of carbon dioxide, a daily production rate of 0·15 mg. of each of these fractions is obtained. For the protein, this quantity is about the same as the daily increase found by Griffiths *et al.*[7] and Turner[8] for two varieties of apple in storage.

An additional point of interest raised by the results in Fig. 4 is the increase of malic acid production by fixation to a rate exceeding the net loss of acid in air as the concentration of external carbon dioxide is raised to 5%. The effect of this consideration on the acid metabolism of the McIntosh apple stored under high concentrations of carbon dioxide is being investigated in these Laboratories.

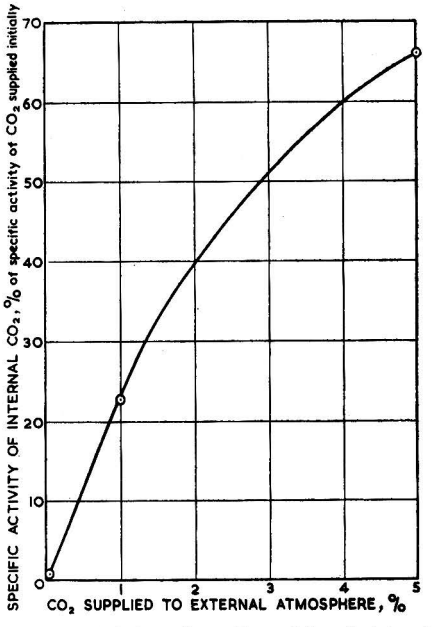


FIG. 3.—*Variation of specific activity of internal carbon dioxide with concentration of carbon dioxide supplied to external atmosphere*

*Seasonal variation of the fixation rate.*—Fig. 5 shows that the specific activity of the internal carbon dioxide of the apples at 22°, when 5% of carbon dioxide was initially supplied to the external atmosphere, rapidly reached 28% of the initial specific activity of the external carbon dioxide, no doubt largely as a result of the initial vacuum injection employed, and remained at this level throughout the 18 hours of exposure. By assuming this factor to remain fairly constant during the period covered by these determinations (September to March), the results of the rate determinations were calculated as in the previous section. The results are plotted in Fig. 6, with mean straight lines for the pre- and post-harvest periods.

The fixation activity rose steeply to a maximum at the time of harvest, and was

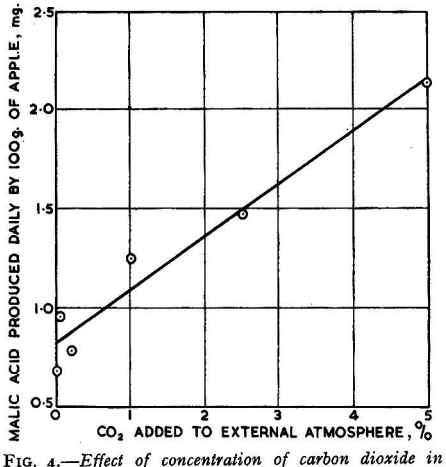


FIG. 4.—*Effect of concentration of carbon dioxide in external atmosphere on production of malic acid by fixation of carbon dioxide at* 0°

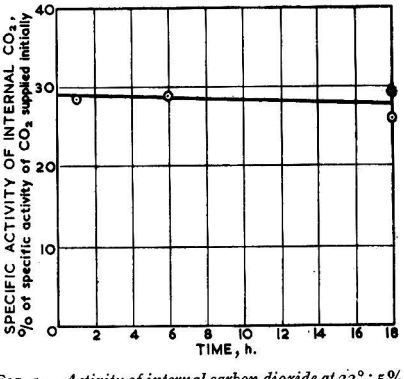


FIG. 5.—*Activity of internal carbon dioxide at* 22°; 5% *of carbon dioxide supplied to the external atmosphere*

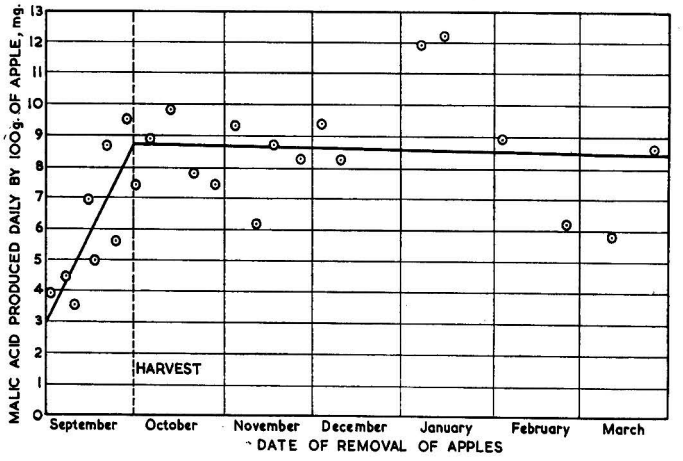


FIG. 6.—*Production of malic acid by fixation of carbon dioxide, 1952–1953, at 22°, with 5% of carbon dioxide initially supplied to the external atmosphere*

maintained at a high level throughout the subsequent storage-life. It is interesting to note that the rise at harvest is concurrent with the respiratory climacteric as determined on apples from the same source. This could be explained by the probable increase of internal carbon dioxide, or it may be indicative of basic metabolic changes. The increased scatter of the points after December may have been due to the incidence, in some of the samples, of the breakdown that was prevalent towards the end of the storage season in question. On the other hand, the apparent maximum in January may have been a genuine reflection of the metabolic changes leading to the increased production of ethylene and other volatiles observed at this time in these Laboratories, although the results in this region are too sparse for any great significance to be ascribed to this variation from the mean.

The highest rates of daily production of malic acid, 8–9 mg., shown in Fig. 6, were determined at 22°, in an atmosphere containing 5% of carbon dioxide. The hypothesis is suggested that in the pre-harvest period the fruit on the tree might be subjected to certain environmental conditions, such as higher temperature, which might result in high respiratory activity. This might increase the concentration of internal carbon dioxide to a point where fixation rates would exceed those found under experimental conditions. Rates of production of malic acid of about 10 mg. daily per 100 g. of apple would thus be possible, representing a significant contribution to the accumulation of acid in the ripening fruit, which reaches a maximum of about 1 g. per 100 g. of apple in the McIntosh variety.

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# THE ISOLATION OF AN ANTHOCYANIN AND CHLOROGENIC ACID FROM CANNED VICTORIA PLUMS

By T. L. PARKINSON

A method is described by which chlorogenic acid and cyanidin-3-monoglucoside are isolated from the liquid portion of canned Victoria plums, so that they may be used in studies on the rate of corrosion of cans of fruit. In the first stage, these substances are separated from all other constituents by adsorption on activated alumina, followed by elution with malic acid solution. In the second stage they are isolated from each other by partition chromatography on a column of cellulose powder. Paper chromatography and absorption spectrophotometry were the main techniques used for the identification of these substances. It appears that the spectrophotometric characteristics of liquors from canned Victoria plums are due mainly to the anthocyanin in the visible region and to chlorogenic acid in the ultra-violet region. The presence of quinic acid in canned plums has also been demonstrated.

## Introduction

It has long been maintained that some of the minor constituents of canned fruits act as accelerators or inhibitors of the electrochemical process of corrosion that takes place inside the can. In a lacquered can this process leads to dissolution of iron and evolution of gaseous hydrogen, resulting in either distortion (' hydrogen swell ') or perforation of the cans. Theoretical aspects of the corrosion process have been summarized and discussed by Hirst & Adam.[1] It has been postulated that substances that act as corrosion accelerators do so by acting as hydrogen acceptors, and some authors have claimed that anthocyanin and other natural pigments function in this way. Kohman[2] reported that the rate of corrosion of cans of peeled apples was accelerated by the addition of ' Logwood Violet ' and ' Yellow Flavine '. These are commercial preparations of haemotoxylin and quercetin, respectively, neither of which is an anthocyanin. There appears to be no record of any studies on the specific effect of an anthocyanin on the rate of corrosion of cans of fruit. Some authors[2, 3] have noted that the rate of corrosion is greater if a larger proportion of coloured parts (e.g. skins) of fruit is present or if a more highly pigmented variety is canned.

It was considered that valuable information might be obtained by isolating a naturally occurring anthocyanin from a particular fruit and studying its influence on the rate of corrosion of steel or tinplate exposed to fruit syrups or solutions of a fruit acid. The fruit chosen for this study was Victoria plums, and a method was evolved by which a solution of the anthocyanin in dilute malic acid was obtained. As malic acid is the major acid present in plums, the solution obtained was in a very convenient form for studying its effect on corrosion. The process, which is described below, also separated another constituent, later identified as chlorogenic acid.

Robinson & Robinson[4] state that the skins of the large blue Californian plum contain cyanidin-3-pentoseglycoside, but that those of the red South African plum and the light-red Gaviota plum contain cyanidin-3-glycoside. These authors say that this contrast between blue and red plums seems to hold generally. Scott-Moncrieff[5] also reports that the anthocyanin in certain varieties of plum is a cyanidin glycoside.

The occurrence of chlorogenic acid in fruit tissue has been established.[6] Johnson, Foreman & Mayer[7] observed that extracts of Italian prunes had a relatively high ultra-violet absorption at 322–324 m$\mu$, which they attributed to the presence of chlorogenic acid.

## Experimental

1. *Separation of anthocyanin and chlorogenic acid from the liquid portion of canned Victoria plums*

Since it is most probable that any accelerators or inhibitors of corrosion will be in the liquid portion of a pack, it was decided to use the syrup from canned plums as starting material.

The method of Nebelsky *et al.*,[8] in which fruit anthocyanins are isolated by adsorption on alumina from an *iso*butanol extract, appeared to offer a satisfactory means of isolating the pigment, especially as it is readily removed from the column by eluting with dilute citric acid. It was considered that, if malic acid, which is the major acid constituent of Victoria plums, were used in place of citric acid, the eluate fraction containing the anthocyanin in dilute malic acid solution would be very suitable for use in experiments on corrosion.

It was found that the anthocyanin could be isolated by this method, but examination, by means of paper chromatography, of the fraction of eluate containing the anthocyanin revealed the presence of another substance, which gave an intense blue fluorescence when

examined in ultra-violet light. This substance was later identified as chlorogenic acid. Attempts to separate the two substances by varying the conditions of elution from the alumina column were unsuccessful, nor could they be separated by the use of ion-exchange resins. A satisfactory separation was eventually achieved by means of partition chromatography on a column of cellulose powder, using ethyl acetate to remove the chlorogenic acid and then eluting the anthocyanin with a mixture of *n*-butanol and malic acid. The coloured fraction from the latter elution was mixed with an equal volume of light petroleum, when the pigment passed quantitatively into the small acidic aqueous phase (cf. Robinson & Robinson[4]). Examination of this solution by paper chromatography in *n*-butanol–acetic acid–water (4 : 1 : 5) showed that the anthocyanin was free from chlorogenic acid. The chlorogenic acid could be recovered from the ethyl acetate eluate by adsorption on activated alumina, followed by elution with 1% malic acid solution.

*Details of method of separation.*—The liquid contents of a can of Victoria plums are separated by straining, and are then mixed in a Waring Blendor with 10 g. of malic acid, 40 g. of sodium chloride and an equal volume of *iso*butanol. The mixture is allowed to separate and the top (*iso*butanol) layer removed. The clear portion of this layer is decanted and the remainder separated further by centrifuging.

When several cans of plums have been treated in this way, the combined *iso*butanol layers are passed through a column of activated alumina (Aluminium Oxide for Chromatographic Analysis, Savory & Moore), 60 cm. in height and 2 cm. in diameter. A purple band separates and slowly moves down the column. When this band is approaching the lower end of the column, addition of *iso*butanol extracts is stopped, and the column is eluted with 1% malic acid solution. The purple fraction of the eluate is collected. This fraction contains both the anthocyanin and the chlorogenic acid. The spent column of alumina is then dried and incinerated at 600° for a few hours, after which it may be used again.

Separation of the two pigments is performed on a column of cellulose pulp. A slurry of Whatman's cellulose powder in ethyl acetate is poured into a tube of 4½-cm. diameter and allowed to settle; the settling process is assisted by tamping with a flat-ended glass rod. When a column 30 cm. high has been prepared, the supernatant ethyl acetate is allowed to drain until its level is just below the top of the column. A suitable volume of the purple fraction is allowed to soak into the cellulose at the top of the column. The column is then washed with about 200 ml. of ethyl acetate. This removes the chlorogenic acid, which may be recovered by passing the ethyl acetate effluent through an alumina column and eluting this with 1% malic acid, following the downward progress of the fluorescent band in ultra-violet light. The cellulose column is then washed with the upper layer of a mixture of equal parts of *n*-butanol and 1% malic acid solution. The red zone then passes down the column and is collected separately as it emerges. To this red solution is added at least an equal volume of light petroleum (b.p. 40–60°). This causes the pigment to pass quantitatively into the lower acidic aqueous layer, which separates when the two solvents are mixed. This layer is drawn off and contains the anthocyanin in dilute malic acid solution. The cellulose column is washed with ethyl acetate and is then ready for another separation.

*2. Identification of the anthocyanin*

Identification of the natural pigments of fruits and flowers has been much simplified by the introduction of paper chromatography. Bate-Smith[9] and Bate-Smith & Westall[10] effected clear separations of anthocyanins in *n*-butanol–acetic acid–water (4 : 1 : 5) and *m*-cresol–acetic acid–water (50 : 2 : 48). They also obtained good separations of the parent anthocyanidins in *n*-butanol–2N-hydrochloric acid (1 : 1).

The malic acid extract of the anthocyanin from Victoria plums was hydrolysed by heating at 100° for 15 minutes with a few drops of concentrated hydrochloric acid. The mixture was then shaken with pentyl alcohol, which quantitatively extracted the coloured anthocyanidin. The aqueous layer, containing the sugar moiety of the anthocyanin, was subsequently examined to determine the nature of the sugar (see below).

(*a*) *Identification of the anthocyanidin.*—Small portions of the anthocyanidin layer were spotted on to a sheet of Whatman's No. 3 filter paper. The chromatograms were developed with the upper layer of the *n*-butanol–2N-hydrochloric acid mixture recommended by Bate-Smith.[9] Elliptical red spots, changing to purple when exposed to ammonia vapour, were observed with $R_F$ values of 0·55. Although it was expected that the anthocyanidin would turn out to be cyanidin, the $R_F$ value obtained was significantly lower than that of 0·69 given by Bate-Smith[9] for cyanidin in the same solvent; the only anthocyanidin for which Bate-Smith[9] gives an $R_F$ value close to 0·55 is malvidin (0·53). It is generally accepted that

$R_F$ values may vary according to conditions, and that reliance should not be placed on published figures as the sole criterion of identity. Consequently it was decided to obtain some authentic samples of the two anthocyanidins in question so that they could be compared directly with that from the plum anthocyanin.

A known source of malvidin galactoside (primulin) is *Primula sinensis*. Through the kindness of Dr. D. Lewis, of the John Innes Horticultural Institution, flowers of this species were made available, as well as a sample of pure malvidin. In addition, a solution of cyanidin was obtained by hydrolysis of chrysanthemin extracted from crimson carnations (see below). Pure malvidin and the anthocyanidins obtained from plums, carnations and primulas were all run on the same chromatogram, with *n*-butanol–2N-hydrochloric acid as solvent. The spots obtained were very elongated, but showed clearly that the anthocyanidins from plums and carnations had $R_F$ values very close to each other but quite different from those of primula anthocyanidin and pure malvidin. More compact and better defined spots were obtained when the solvent mixture was modified by the addition of ethanol, the proportions of the ingredients being *n*-butanol (18 parts) : ethanol (2) : 2N-hydrochloric acid (10). $R_F$ values obtained when this solvent (upper layer) was used were : anthocyanidin obtained from Victoria plums 0·63, anthocyanidin obtained from crimson carnations 0·64, anthocyanidin obtained from *Primula sinensis* 0·43 and pure malvidin 0·46.

That the anthocyanidin from plums was cyanidin and not malvidin was further supported by the $R_F$ values in *m*-cresol–acetic acid (see below) of the anthocyanins from plums, carnations, *Primula sinensis* and grapes, which are low (0·2–0·3) for the two first-named and high (0·7–0·8) for the last two. Bate-Smith's[9] figures show that cyanidin derivatives have low and malvidin derivatives high $R_F$ values in this solvent mixture.

The identity of the anthocyanidin from plums with cyanidin was confirmed by applying the colour tests of Robinson & Robinson.[4] Solutions in pentyl alcohol (over three volumes of 0·5% hydrochloric acid) of the plum anthocyanidin, and of cyanidin obtained from carnations, both required six times their volume of benzene to decolorize the upper layer. Pentyl alcoholic solutions (over water) of both substances gave a red-violet colour with sodium acetate, and this was changed to blue or blue-green on addition of a drop of ferric chloride solution. A rose colour was obtained with the cyanidin reagent.

(*b*) *Identification of the sugar.*—The aqueous solution left after extracting the hydrolysed anthocyanin with pentyl alcohol was desalted by passing it successively through Zeo-Karb 215 and Deacidite B. The desalted extract was concentrated by warming *in vacuo*. A small aliquot of the concentrated solution was subjected to paper chromatography in *n*-butanol–acetic acid–water (4 : 1 : 5). Spraying the dried chromatogram with silver nitrate, by means of the technique described by Trevelyan, Proctor & Harrison,[11] revealed only one spot, which had an $R_F$ value of 0·18. This suggested that the sugar was either glucose or galactose. These two sugars are not easy to differentiate by paper chromatography, as their $R_F$ values in most solvents are too close to allow any clear distinction between them. However, separation (of these sugars) is possible by the method of Jermyn & Isherwood,[12] in which the solvent is allowed to drip off the lower edge of a sheet of paper for a few days, so that the sugar spots move to a much greater distance from their starting points. Accordingly, 40-$\mu$l. aliquots of the concentrated, de-ionized hydrolysate were spotted on to a sheet of Whatman's No. 54 filter paper, together with spots (15 $\mu$l. each) of 2% solutions of glucose and galactose. The bottom edge of the sheet was serrated, as recommended by Jermyn & Isherwood,[12] and the chromatogram was run for two days in a solvent mixture consisting of ethyl acetate–acetic acid–water (3 : 1 : 3). Jermyn & Isherwood[12] recommend this solvent as giving an adequate separation of glucose and galactose. A similar chromatogram was run for three days. The chromatograms were dried and treated with the silver nitrate reagent by the method of Trevelyan *et al.*,[11] and it was observed that the spots had travelled the following distances :

| | After 2 days | After 3 days |
|---|---|---|
| Sugar from anthocyanin | 31·7 cm. | 39·0 cm. |
| Glucose | 31·2 cm. | 38·7 cm. |
| Galactose | 27·8 cm. | 35·0 cm. |

That the sugar was dextrose was confirmed by preparing the osazone from the de-ionized hydrolysate. This proved to be identical in appearance and melting point (205°) with the osazone prepared from pure dextrose, and different from galactosazone (m.p. 188°).

(*c*) *Identification of the anthocyanin.*—It having been established that the anthocyanin was a cyanidin-glucoside, the next step was comparison, by paper chromatography, spectrophotometry and colour reactions, of the anthocyanin from Victoria plums with authentic samples

of the different glucosides of cyanidin known to occur in nature. Robinson & Robinson[4] give a list of sources of many anthocyanins, and a similar list is given by Sannié & Sauvain.[13]

Extracts of the following anthocyanins were prepared: chrysanthemin (cyanidin-3-monoglucoside) from crimson carnations; cyanin (cyanidin-3 : 5-diglucoside) from (*a*) red roses and (*b*) blue cornflowers. These extracts, together with the anthocyanin isolated from plums, were subjected to paper chromatography in the two solvent systems recommended by Bate-Smith.[9] The $R_F$ values obtained are recorded in Table I.

**Table I**

*Comparison of $R_F$ values of the anthocyanin isolated from canned Victoria plums with those of known anthocyanins*

| | $R_F$ values in | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *n*-butanol–acetic acid–water (40 : 10 : 50) | | | *m*-cresol–acetic acid–water (50 : 2 : 48) | | | | | |
| | Experiment 1 | | Experiment 2 | Experiment 1 | | | Experiment 2 | | |
| Anthocyanin from plums | 0·31 | 0·29 | 0·26 | 0·22 | 0·23 | 0·24 | 0·23 | 0·24 | 0·22 |
| Chrysanthemin from carnations | 0·30 | 0·29 | 0·25 | 0·21 | 0·25 | 0·24 | 0·23 | 0·22 | 0·21 |
| Cyanin from roses | 0·19 | 0·18 | 0·14 | 0·09 | 0·09 | | 0·10 | | |
| Cyanin from cornflowers | | | 0·15 | | | | | | |

When the dried chromatograms were exposed to fumes of ammonia, the spots derived from plums and carnations became purple and those from roses and cornflowers became bright blue. It was therefore concluded that the anthocyanin isolated from canned Victoria plums was cyanidin-3-monoglucoside. Confirmation was obtained by carrying out the following colour tests of Robinson & Robinson[4]: (i) On addition of sodium acetate, the plum anthocyanin and chrysanthemin gave a violet colour, and cyanin a blue colour; (ii) the ferric chloride test produced a red-violet colour with plum anthocyanin and chrysanthemin, and blue with cyanin; (iii) addition of sodium carbonate produced blue-violet colours with plum anthocyanin and chrysanthemin, and a bright-blue colour with cyanin.

A malic acid extract of the anthocyanin from Victoria plums was adjusted to pH 3·0 (which was the pH of the original syrup from the canned plums) with sodium malate solution. The absorption spectrum of this solution in the visible region was recorded from measurements taken in the spectrophotometer described by Richard.[14] The absorption spectra of a similar extract, in both ultra-violet and visible regions, were determined with a Unicam quartz spectrophotometer SP.500. These spectra are shown in Figs. 1 and 2, respectively.

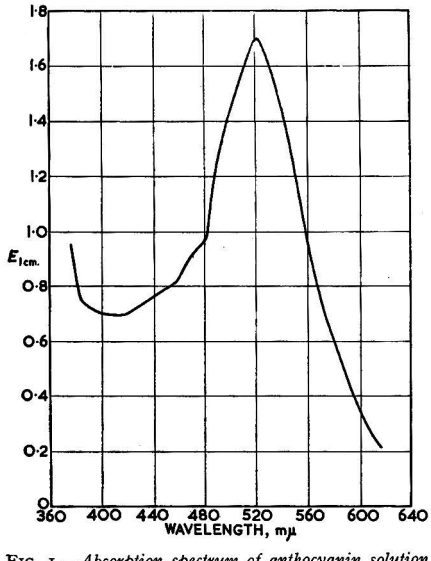


FIG. 1.—*Absorption spectrum of anthocyanin solution from canned Victoria plums; pH = 3·0*

(*d*) *Identity of the anthocyanin in raw and canned plums.*—It was considered desirable to confirm that raw Victoria plums also contain cyanidin-3-monoglucoside, and that the presence of this compound in canned plums is due solely to extraction, and is not derived by hydrolysis of a more complex glycoside. Accordingly, the

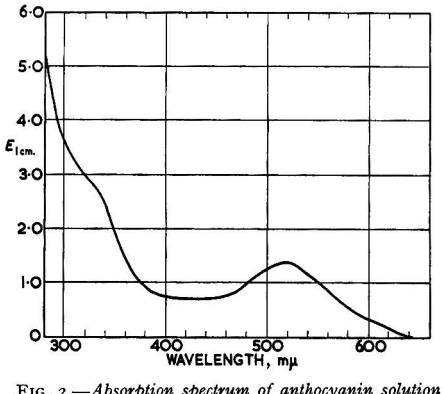


FIG. 2.—*Absorption spectrum of anthocyanin solution from canned Victoria plums; pH = 3·0*

anthocyanin was isolated from raw Victoria plums and compared with that from the canned plums by paper chromatography. The results obtained are given in Table II, which also records a comparison between the anthocyanins obtained from canned Victoria plums and canned Purple Pershore plums. It will be seen that raw and canned Victoria plums and canned Purple Pershore plums all contain the same anthocyanin.

**Table II**

*$R_F$ values of anthocyanins extracted from raw and canned Victoria plums and from canned Purple Pershore plums*

| Solvent mixture | | Canned Purple Pershore plums | Canned Victoria plums | Raw Victoria plums | Chrysanthemin from carnations |
|---|---|---|---|---|---|
| *n*-Butanol–2N-HCl (1 : 1) | Experiment 1 | 0·20 | 0·20 | | |
| | ,, 2 | 0·20 | 0·20 | | |
| *n*-Butanol–acetic acid–water (40 : 10 : 50) | Experiment 1 | 0·24 | 0·23 | | 0·22 |
| | ,, 2 | 0·22 | 0·23 | | |
| *n*-Butanol–acetic acid–water (60 : 15 : 25) | Experiment 1 | | 0·36 | 0·35 | |
| | ,, 2 | | 0·38 | 0·38 | |
| | ,, 3* | | 0·27 | 0·26 | 0·25 |
| *m*-Cresol–acetic acid–water (50 : 2 : 48) | Experiment 1 | | 0·22 | 0·22 | 0·21 |
| | ,, 2 | | 0·22 | 0·21 | 0·21 |

* In this instance the solvent had already been used for experiments 1 and 2 on the previous day, and therefore was not freshly prepared. This probably accounts for the different $R_F$ values in Experiment 3

3. *Identification of chlorogenic acid*

The substance that accompanied the anthocyanin, when the anthocyanin was eluted from the alumina column by malic acid solution, was detected by the strongly fluorescent spot ($R_F$ 0·48–0·56) that appeared when the eluate was subjected to paper chromatography in *n*-butanol–acetic acid–water (4 : 1 : 5). In ultra-violet light this spot displayed an intense blue fluorescence, which was changed to duck-egg green when the chromatogram was exposed to ammonia vapour; in daylight, exposure to ammonia showed up this substance as a yellow spot. When the chromatogram was sprayed with neutral 1% ferric chloride solution a grey-blue spot appeared, and with Benedict's solution a bright-yellow spot was obtained. When the chromatogram was sprayed with 1% sodium nitrite dissolved in 10% acetic acid a yellow spot appeared, which turned brick-red when subsequently sprayed with N-potassium hydroxide solution. This spray reaction was developed by Roberts & Wood[15] for the detection of chlorogenic acid. A small amount of crystalline chlorogenic acid was obtained from Dr. D. Dickinson. This was dissolved in 1% malic acid solution and was subjected to chromatography on the same sheet of paper as the material isolated from Victoria plums. The two substances had identical $R_F$ values, had the same appearance in ultra-violet light, and gave similar colours with the reagents described above.

A solution of the substance in 1% malic acid, obtained by the process described above, was adjusted to pH 3·0 with sodium malate solution. A solution of crystalline chlorogenic acid in 1% malic acid was similarly adjusted to pH 3. The ultra-violet absorption spectra of these solutions were measured in a Unicam quartz spectrophotometer, and are shown in Fig. 3. The two spectra have been made to coincide at the wavelength (325 m$\mu$) showing maximum optical density, and it will be seen that they bear a close resemblance. They also closely resemble spectra of chlorogenic acid published by other authors.[16, 17]

Hulme[16] used paper chromatography to show the presence of caffeic acid and quinic acid formed by hydrolysis of chlorogenic acid. The solution of chlorogenic acid obtained from plums by the method described above was hydrolysed by heating for 15 minutes with 13% potassium hydroxide at 20°, and neutralized to pH 4·0. This hydrolysate was passed first through a column of Zeo-Karb 215 and then through a column of Deacidite E, on which the free organic acids were absorbed. The latter column was washed with distilled water and then eluted with N-sodium carbonate solution. This eluate contained the sodium salts of the organic acids, and these were converted into the free acids by passing the solution through a second column of Zeo-Karb 215. The effluent from this last ion-exchange treatment was subjected to paper chromatography on Whatman No. 3 filter paper, using the organic layer of a mixture of equal parts of *n*-butanol and 2M-formic acid, as recommended by Lugg & Overell.[18] Spots of pure specimens of caffeic acid and quinic acid were placed on the same sheet of paper. The acids from hydrolysed chlorogenic acid revealed a blue fluorescent spot

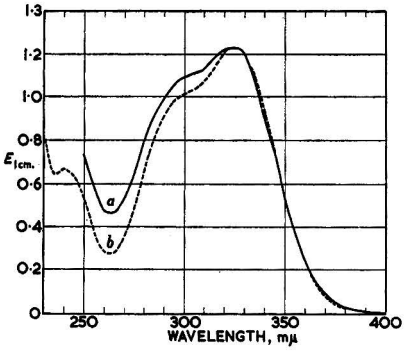


FIG. 3.—*Ultra-violet absorption spectra of*

(*a*) Solution of chlorogenic acid isolated from canned Victoria plums
(*b*) Pure chlorogenic acid in malic acid–malate buffer solution; pH = 3·0

of $R_F$ 0·79 when examined in ultra-violet light, and also showed a non-fluorescent acidic spot of $R_F$ 0·17 when sprayed with bromophenol blue or 2 : 6-dichlorophenolindophenol. Caffeic acid was revealed by ultra-violet light as a blue fluorescent spot of $R_F$ 0·79, and quinic acid appeared as a non-fluorescent acidic spot of $R_F$ 0·16. The spots of $R_F$ 0·79 from caffeic acid and hydrolysed chlorogenic acid each gave grey-blue colours when sprayed with neutral 1% ferric chloride solution. The spots of $R_F$ 0·16–0·17 from pure quinic acid and from hydrolysed chlorogenic acid each reduced alkaline silver nitrate solution.

### 4. *Detection of quinic acid in the liquid portion of canned plums*

The method described in the preceding paragraph for isolating organic acids from hydrolysed chlorogenic acid by means of ion-exchange resins was first developed with the object of identifying the organic acids present in the liquid portion of canned Victoria plums. Similar procedures have been published by other authors.[19, 20]

A solution of the acids from canned plums, obtained by this method, was examined by paper chromatography in *n*-butanol–2M-formic acid. It was found advisable to set aside the dried chromatogram at room temperature for 24 hours before spraying with the indicator, as recommended by Lugg & Overell.[18] It was apparent that the major acid constituent was malic acid, which formed a large spot with an $R_F$ value (0·50) identical with that of pure malic acid run on the same chromatogram. A smaller, but definite spot of $R_F$ 0·20–0·22 was also present and could not at first be identified. Hulme & Swain[21] reported a spot of similar $R_F$ value in extracts of apples and plums, and later Hulme[22] identified this substance as quinic acid. Pure quinic acid gave a spot with an $R_F$ value (0·20–0·22) identical with that of the slower moving spot from the plum-syrup extract when both were run on the same chromatogram and sprayed with an acid–base indicator. Further, both quinic acid and the substance in the plum-syrup extract gave brown spots when sprayed with alkaline silver nitrate according to Trevelyan *et al.*[11] This reagent also revealed another spot ($R_F$ 0·11), which appeared to correspond to galacturonic acid.

### 5. *The contribution of the anthocyanin to the red colour of plums*

Figs. 4 and 5 record the absorption spectra, in the visible region, of the clear liquors strained from the following samples of canned plums: (i) Victoria plums of good colour, picked from a sunny situation [Fig. 4, curve (*a*)]. (ii) Victoria plums, pale in colour, picked from a shaded position on the same tree as (i) [Fig. 4, curve (*b*)]. (iii) Purple Pershore plums, canned whole [Fig. 5, curve (*a*)]. (iv) Purple Pershore plums, canned with the skins removed [Fig. 5, curve (*b*)].

No artificial colouring was used in samples (i) and (ii); samples (iii) and (iv) were commercial packs and may have contained a little added colour. All samples were examined after having been stored at ordinary temperatures for only a few days. Comparing these spectra with those of Fig. 1, it will be seen that it is the anthocyanin that is mainly responsible for the red colour of the canned plums. It also appears that the anthocyanin is located in the skins, where it is probably formed as the result of some photolytic reaction.

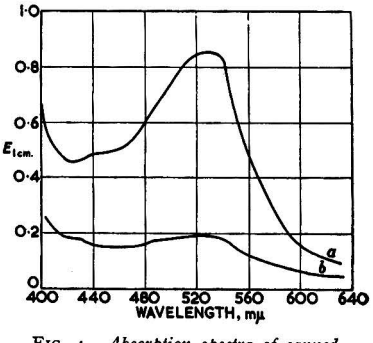


FIG. 4.—*Absorption spectra of canned Victoria plums*

(*a*) Coloured fruit (*b*) Pale fruit

6. *Removal of anthocyanin from canned plums by ion-exchange*

It had been observed that when the strained liquor from canned plums was passed through a column of Zeo-Karb 215, the resulting effluent was much paler in colour, even after it had been adjusted to the original pH, than the inflowing liquid. In fact, the first runnings of the effluent were almost colourless, but, as the liquid continued to flow through the column, the effluent gradually became pinker. This phenomenon has also been observed by Bradfield & Flood.[23] The absorbed pigment was removed by washing the Zeo-Karb column with, successively, distilled water, N-sodium carbonate solution and finally 2N-hydrochloric acid. Figs. 6 and 7 show the absorption spectra (in the visible region) of the following solutions: (i) Liquid from canned plums before passing through Zeo-Karb 215 [Fig. 6, curve (*a*)]; (ii) liquid from canned plums after passing through Zeo-Karb 215 [Fig. 6, curve (*b*)]; (iii) coloured solution of pigment adsorbed on Zeo-Karb 215 and subsequently removed by alkaline and acid washing (Fig. 7).

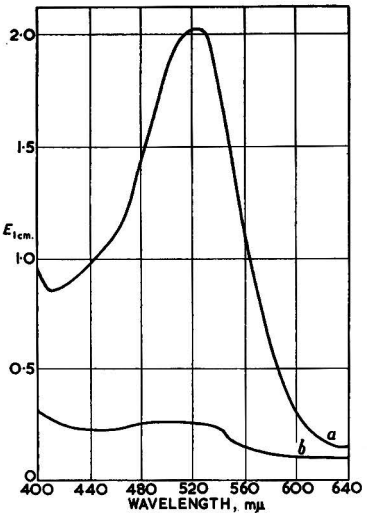


FIG. 5.—*Absorption spectra of liquors from canned Purple Pershore plums*
(*a*) Canned whole plums (*b*) Canned peeled plums

These results show clearly that the anthocyanin can be adsorbed on the cation-exchange resin. This was confirmed by running an aliquot of solution (iii) on a paper chromatogram, together with a sample of the anthocyanin solution obtained by the *iso*butanol–alumina–malic acid–cellulose treatment described above. Red spots, turning purple when exposed to ammonia vapour, and having the same $R_F$ values, were obtained from both samples.

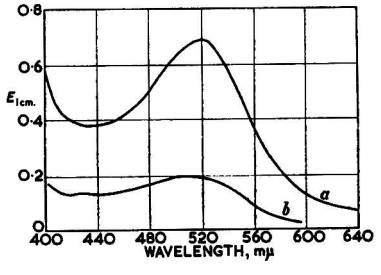


FIG. 6.—*Absorption spectra of liquor from canned Victoria plums*
(*a*) before and (*b*) after passage through a column of Zeo-Karb 215

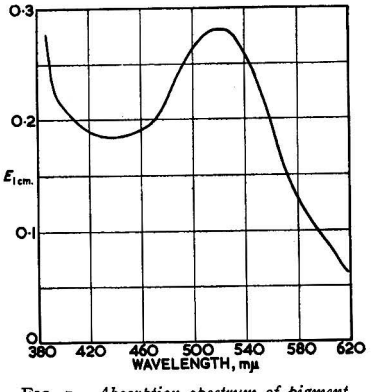


FIG. 7.—*Absorption spectrum of pigment removed from canned Victoria plums by Zeo-Karb 215*

7. *Effect of storage on the anthocyanin content of canned plums*

Fig. 8 shows the absorption spectra (in the visible region) of the following samples: (i) Victoria plums (in syrup) bottled in Kilner jars and stored at − 15° [curve (*a*)]; (ii) canned Victoria plums stored at room temperature for 25 weeks [curve (*b*)]; (iii) canned Victoria plums stored at 37° for 25 weeks [curve (*c*)].

It is obvious from these curves that the anthocyanin disappears from the liquid portion of canned plums during storage at normal or elevated temperatures. At the higher temperature the change is masked by an increase in general absorption, but it would seem that the

disappearance of anthocyanin is more rapid at 37° than at room temperature. It is probable that the anthocyanin undergoes chemical change, but the possibility of adsorption on either the solid portion of the fruit or the interior surface of the can must not be ignored. It is difficult to assess the extent of these losses, owing to the large variation in anthocyanin content among individual plums, and hence among individual cans, in the same batch (see, for example, Fig. 4). It is proposed to study this change in more detail by canning and bottling a well-mixed bulk sample of the liquor from freshly canned plums and then examining the absorption spectra of samples at intervals during storage at two different temperatures.

8. *Chlorogenic acid and the ultra-violet absorption spectra of canned plums*

Fig. 9 gives the ultra-violet absorption spectra (measured in a Beckmann spectrophotometer) of samples of liquors from Victoria plums that had been preserved and stored as follows: (i) Bottled in syrup in a Kilner jar and stored at − 15° [curve (*a*)]; (ii) canned in syrup and stored at 37° for 28 weeks [curve (*b*)]; (iii) as (i) and passed through a column of Zeo-Karb 215 [curve (*c*)].

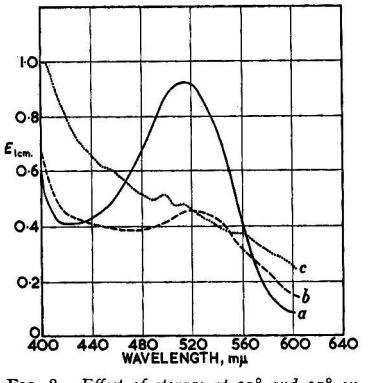


FIG. 8.—*Effect of storage at 20° and 37° on the absorption spectrum (visible region) of canned Victoria plums*

(*a*) Stored in frozen state
(*b*) Stored at room temperature for 25 weeks
(*c*) Stored at 37° for 25 weeks

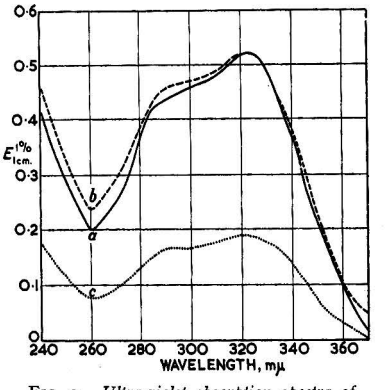


FIG. 9.—*Ultra-violet absorption spectra of liquors from canned Victoria plums*

(*a*) Freshly canned
(*b*) Stored at 37° for 28 weeks
(*c*) Sample (*a*) after passage through Zeo-Karb 215

In all instances the liquor was adjusted to pH 3·0, if necessary, and diluted with distilled water to 100 times its original volume.

Comparison of these spectrum curves with those of Fig. 3 reveals that chlorogenic acid is the main ultra-violet-absorbing substance leached into the syrup during the canning of Victoria plums. Fig. 9 also reveals that a large proportion of the chlorogenic acid can be removed from canned Victoria plums by passing the liquor through a column of the cation-exchange resin Zeo-Karb 215.

**Acknowledgments**

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# THE COMPONENT FATTY ACIDS OF BOVINE MAMMARY-GLAND FAT

By G. A. GARTON

The component acids present in the fat of the secretory tissue of the udders of a lactating and a non-lactating cow were determined. The fat of the lactating gland contained about half as much of the lower saturated acids (butyric–capric) as a typical milk-fat, with a compensating increase in the proportions of stearic and palmitic acid; all the minor unsaturated acids of milk fat were detected. Although the fat of the non-lactating gland contained small amounts of the minor saturated and unsaturated acids characteristic of milk fat, it differed from lactating-gland fat and also from bovine depot-fat in the relative amounts of the major component acids; palmitic acid comprised more than 40% of the total acids of this fat.

As part of a study on the relationship between blood glycerides, depot-fat and milk-fat formation in the cow it was considered desirable to determine the fatty-acid composition of the fat of the secretory tissue of the udder, although it was recognized that it would not be possible to extract separately the lipids present in the alveolar cells and their lumena, in the connective tissue and any secreted milk-fat remaining in the lactiferous ducts. As far as is known, previous work on bovine mammary-gland fat has been restricted to the determination of analytical constants. Thus Petersen, Palmer & Eckles[1] reported that the fat of four lactating bovine mammary glands was intermediate in character between butter fat and depot fat as judged by saponification equivalent, iodine value and Reichert–Wollny number, whereas the fat from three non-lactating glands resembled depot fat in its analytical characteristics. Kelly & Petersen[2] found a correlation between the amount of free fatty acid in bovine udder fat and the state of lactation, about twice as much free acid occurring in the fat from the actively secreting glands as in the non-lactating glands. The Reichert–Meissl value of the mammary-gland fat of two lactating cows was found by Gowen & Tobey[3] to be 11·8 and 14·4, as compared with 0·0–0·4 for the gland fat from six non-lactating animals.

## Experimental

*Extraction of fats.*—The udders were removed from two cows immediately after slaughter; one animal had just ceased lactating and the second was lactating when killed. Both udders were suspended in a cool room overnight, when a small amount of residual milk in the lactating udder drained away. The whole of the secretory tissue (1077 g.) from the udder of the non-lactating cow was then dissected out; the secretory tissue (3235 g.) from the left half of the lactating udder was dissected out a month later after having been frozen at — 20° during the intervening time. The mammary-gland tissue was cut into small pieces, blotted with filter paper and then minced in a household mincing-machine.

The minced tissue was extracted with boiling acetone (2·5 l./kg. of tissue) under reflux for 5 h. The acetone was decanted through a sintered-glass filter and the extraction with acetone repeated. Two further 5-h. extractions were carried out with a 3 : 1 (v/v) ethanol–ether mixture (2·5 l./kg. of tissue). The ethanol–ether extracts were a much deeper orange colour than were the acetone extracts. The solvents were removed leaving orange-coloured, solid lipid material, 13·7% of the wet weight of the tissue from the non-lactating cow and 7·4% from the lactating cow. During the removal of the solvents of the extract from the tissue of the lactating animal a flocculent precipitate of protein appeared and was filtered off; this protein gave the usual qualitative tests for caseinogen.

The general analytical characteristics of the mammary-gland fats are recorded in Table I; carotene estimations were made by method A of Morton, Lord & Goodwin.[4]

**Table I**

*Analytical characteristics of bovine mammary-gland fats*

| | Source of fat | |
|---|---|---|
| | Secretory tissue of non-lactating cow | Secretory tissue of lactating cow |
| Iodine value | 41·5 | 42·7 |
| Saponification equivalent | 274·0 | 270·0 |
| Free fatty acid (as oleic, %) | 1·2 | 1·9 |
| Unsaponifiable matter, % | 1·85 | 2·0 |
| Carotene, μg./g. | 10 | 55 |

*Component fatty acids of the mammary-gland fats*

Both fats were saponified by refluxing for two hours with excess of ethanolic potassium hydroxide. The fatty acids were then separated as described below into three groups: (1) steam-volatile acids, (2) non-volatile acids soluble in acetone at — 40° and (3) non-volatile acids insoluble in acetone at — 40°.

*Steam-volatile acids.*—As described for cow-milk fats,[5–7] the ethanol was completely removed from the soaps and the acids were liberated with sulphuric acid in a steam-distillation apparatus. Distillation was continued for 5·5 h. after which the condenser was washed with pure ether and the acids were extracted from the distillate with ether. After being set aside overnight over anhydrous sodium sulphate the ethereal solution was filtered and the ether carefully removed leaving the free fatty acids. The recovered ether and the extracted aqueous distillate were titrated against 0·1N-sodium hydroxide solution to determine the residual acidity, which was calculated as butyric acid.

*Low-temperature crystallization of the acids non-volatile in steam.*—The non-volatile acids were recovered from the steam-distillation apparatus by ether extraction. The ethereal solution was dried over anhydrous sodium sulphate and filtered; removal of the solvent left the fatty acids, which were dissolved in acetone (10 ml./g. of acids) and crystallized for 6 h. at — 40°.

The results of these preliminary separations of the mixed fatty acids are shown in Table II.

*Treatment of steam-volatile acids.*—A portion of the steam-volatile acids was fractionally distilled in a semi-micro fractional distillation apparatus; the equivalents of the fractions were determined as described by Lovern.[8]

*Treatment of solvent-segregated acids.*—Each group of acids was converted into its methyl esters and distilled *in vacuo* through an electrically heated and packed column. The methods used for the fractional distillation of the methyl esters and for the analytical (including spectrophotometric) examination of the ester fractions were those described by Hilditch[9] and Gupta & Hilditch.[10]

The composition of each ester fraction and each steam-volatile acid fraction was then

**Table II**

*Preliminary separation of the fatty acids of the mammary-gland fats*

| Fraction | Description | Weight, g. | Weight, % of total | Iodine value | Sapon. equiv. |
|---|---|---|---|---|---|
| | Lactating gland | | | | |
| V | Volatile in steam | 4·73 | 3·7 | — | — |
| A | Non-volatile in steam, insoluble in ether at − 40° | 59·40 | 46·1 | 5·1 | 261·0 |
| B | Non-volatile in steam, soluble in ether at − 40° | 64·90 | 50·2 | 86·9 | 260·0 |
| | Non-lactating gland | | | | |
| V | Volatile in steam | 0·95 | 1·2 | — | — |
| A | Non-volatile in steam, insoluble in ether at − 40° | 37·8 | 49·3 | 2·6 | 263·0 |
| B | Non-volatile in steam, soluble in ether at − 40° | 38·0 | 49·5 | 69·3 | 263·6 |

calculated by methods described (Hilditch[9]; Gupta & Hilditch[10]), and the percentage composition of each group of acids was thus derived. These compositions and the consequent composition of the total fatty acids in the mammary-gland fats are shown in Tables III and IV.

**Table III**

*Component acids in groups V, A, B, and in the whole fat of the mammary gland of the lactating cow (separation into groups V, A, and B is shown in Table II)*

| Acid | V, %* (3·7%)† | A, %* (46·1%)† | B, %* (50·2%)† | Total | Fatty acids in the whole fat, %(w/w) | Fatty acids in the whole fat, % (mol.) |
|---|---|---|---|---|---|---|
| Butyric | 2·23 | — | — | 2·23 | 2·3 | 6·5 |
| Caproic | 0·90 | — | — | 0·90 | 0·9 | 2·0 |
| Caprylic | 0·46 | — | — | 0·46 | 0·5 | 0·8 |
| Capric | — | — | 0·63 | 0·63 | 0·6 | 0·9 |
| Lauric | — | — | 2·01 | 2·01 | 2·1 | 2·6 |
| Myristic | — | 3·17 | 1·14 | 4·31 | 4·4 | 4·8 |
| Palmitic | — | 27·44 | 3·79 | 31·23 | 32·0 | 31·1 |
| Stearic | — | 12·06 | 1·99 | 14·05 | 14·3 | 12·6 |
| Decenoic | 0·11 | — | 0·06 | 0·17 | 0·2 | 0·3 |
| Dodecenoic | — | — | 0·41 | 0·41 | 0·4 | 0·5 |
| Tetradecenoic | — | — | 0·43 | 0·43 | 0·4 | 0·5 |
| Hexadecenoic | — | — | 5·78 | 5·78 | 5·9 | 5·8 |
| Hexadecadienoic | — | — | 0·38 | 0·38 | 0·4 | 0·4 |
| Octadecenoic | — | 3·05 | 25·56 | 28·61 | 29·3 | 25·8 |
| Octadecadienoic (conjugated) | — | — | 0·65 | 0·65 | 0·7 | 0·6 |
| Octadecadienoic (conjugatable) | — | — | 0·74 | 0·74 | 0·8 | 0·7 |
| Octadecadienoic (non-conjugatable) | — | — | 2·09 | 2·09 | 2·1 | 1·9 |
| Octadecatrienoic | — | — | 0·74 | 0·74 | 0·8 | 0·7 |
| Unsaturated $C_{20}$–$C_{22}$ | — | — | 1·88 | 1·88 | 1·9 | 1·5 |
| Unsaponifiable | — | 0·38 | 1·92 | 2·30 | — | — |

* Component acids as % (w/w) of total acids
† Groups as % (w/w) of total acids

## Discussion

For comparative purposes the composition of the two fats that have been analysed are given in Table V together with published figures for a typical cow-milk fat[11] and a cow depot-fat.[12]

As mentioned earlier, fat extracted from the secretory tissue of the udder represents a mixture originating in the different structural parts of the gland, and includes material from the interalveolar and interlobular connective tissue; the composition of the interalveolar and interlobular fat probably resembles that of depot fat. Nevertheless, interesting differences exist between the fatty acid composition of the fat from the lactating and non-lactating glands and between the fat of the lactating gland and milk fat.

The fat from the lactating gland contains smaller amounts of the saturated acids butyric–myristic than a typical butter-fat, but contains proportionally more palmitic and stearic acids. Of the unsaturated acids, $C_{10}$–$C_{14}$ monoethenoid acids occur to about the same extent as in milk fat, but the amount of hexadecenoic (palmitoleic) acid is considerably greater than that

## Table IV

*Component acids in groups V, A, B, and in the whole-fat of the mammary gland of the non-lactating cow (separation into groups V, A and B is shown in Table II)*

| Acid | V, %* (1·20%)† | A, %* (49·3%)† | B, %* (49·5%)† | Total | Fatty acids in the whole fat, % (w/w) | % (mol.) |
|---|---|---|---|---|---|---|
| Butyric | 0·35 | — | — | 0·35 | 0·4 | 1·2 |
| Caproic | 0·05 | — | — | 0·05 | 0·1 | 0·2 |
| Caprylic | 0·16 | — | — | 0·16 | 0·2 | 0·4 |
| Capric | 0·33 | — | — | 0·33 | 0·3 | 0·4 |
| Lauric | 0·25 | — | 1·33 | 1·58 | 1·6 | 2·1 |
| Myristic | — | 1·97 | 2·10 | 4·07 | 4·1 | 4·7 |
| Palmitic | — | 32·72 | 7·90 | 40·62 | 40·7 | 41·3 |
| Stearic | — | 13·18 | 4·96 | 18·14 | 18·1 | 16·5 |
| Decenoic | 0·06 | — | — | 0·06 | 0·1 | 0·2 |
| Dodecenoic | — | — | 0·35 | 0·35 | 0·4 | 0·5 |
| Tetradecenoic | — | — | 1·05 | 1·05 | 1·1 | 1·3 |
| Hexadecenoic | — | — | 8·83 | 8·83 | 8·8 | 9·0 |
| Hexadecadienoic | — | — | 0·51 | 0·51 | 0·5 | 0·5 |
| Octadecenoic | — | 1·43 | 18·74 | 20·17 | 20·2 | 18·6 |
| Octadecadienoic (conjugated) | — | — | 0·93 | 0·93 | 0·9 | 0·8 |
| Octadecadienoic (conjugatable) | — | — | 1·22 | 1·22 | 1·2 | 1·1 |
| Octadecatrienoic | — | — | 1·26 | 1·26 | 1·3 | 1·2 |
| Unsaturated $C_{20}$–$C_{22}$ | — | — | Trace | Trace | Trace | Trace |
| Unsaponifiable | — | — | 0·32 | 0·32 | — | — |

* Component acids as % (w/w) of total acids
† Groups as % (w/w) of total acids

usually found in milk fats; in addition, the presence of a $C_{16}$ diethenoid acid was detected spectrophotometrically. Oleic acid is the major unsaturated acid of the lactating-gland fat; the amount is of the same order as that found in milk fat by Hilditch & Longenecker[7] and Hilditch & Jasperson.[11] In the present study more $C_{18}$ diethenoid acids are reported than have so far been found in milk fats, although Hilditch & Jasperson[13] found small amounts of non-conjugated and conjugated forms of octadecadienoic acid together with traces of octadecatrienoic acid in cow-milk fat. Polyethenoid $C_{16}$ and $C_{18}$ acids (mainly non-conjugatable dienoic acids) are now known to occur in the depot fats of the hippopotamus (Barker & Hilditch[14]), ox, sheep and elephant (Cama[15]) and probably in the body fats of most other species.

## Table V

*Comparison of the component fatty acids [% (mol.)] of bovine depot-fat, milk and mammary-gland fat*

| Acid | Source of fat: Milk (Hilditch & Jasperson[11]) | Lactating mammary gland (present work) | Perinephric depot-fat (Hilditch & Longenecker[12]) | Non-lactating mammary gland (present work) |
|---|---|---|---|---|
| Butyric | 10·5 | 6·5 | — | 1·2 |
| Caproic | 4·6 | 2·0 | — | 0·2 |
| Caprylic | 1·3 | 0·8 | — | 0·4 |
| Capric | 2·7 | 0·9 | — | 0·4 |
| Lauric | 2·6 | 2·6 | 0·2 | 2·1 |
| Myristic | 9·6 | 4·8 | 3·7 | 4·7 |
| Palmitic | 23·4 | 31·1 | 26·5 | 41·3 |
| Stearic | 9·7 | 12·6 | 23·1 | 16·5 |
| As arachidic | 0·6 | — | 0·7 | — |
| Decenoic | 0·3 | 0·3 | — | 0·2 |
| Dodecenoic | 0·2 | 0·5 | — | 0·5 |
| Tetradecenoic | 1·0 | 0·5 | 0·5 | 1·3 |
| Hexadecenoic | 2·1 | 5·8 | 2·6 | 9·0 |
| Hexadecadienoic | n.d. | 0·4 | n.d. | 0·5 |
| Octadecenoic | 28·6 | 25·8 | 40·4 | 18·6 |
| Octadecadienoic | 1·8 | 3·2 | 1·8 | 1·9 |
| Octadecatrienoic | n.d. | 0·7 | n.d. | 1·2 |
| Unsaturated $C_{20}$–$C_{22}$ | 1·0 | 1·5 | 0·5* | Trace |

* As arachidonic acid
n.d. Not determined

In the fat from the non-lactating gland all the saturated fatty acids found in milk fat and the lactating-gland fat are present, together with all the unsaturated acids except the non-conjugatable octadecadienoic acid that was found in the fat of the lactating gland. Apart from small amounts of the lower steam-volatile acids and the minor unsaturated components, it might have been expected that the fat of the non-lactating gland would approximate more closely to depot fat in the relative proportions of its major component acids than occurred in the fat of the lactating gland. However, this is not so and the amount of palmitic acid [> 40% (mol.)] is very high and the stearic and oleic acid contents are reduced well below the amounts normally found in depot fat; the low content of oleic acid [18·6% (mol.)] is especially noteworthy. Corresponding to the high content of palmitic acid is the unusually high percentage of $C_{16}$ unsaturated acids, mainly palmitoleic.

Although the fat of only one non-lactating gland was examined, a possible explanation of the high content of palmitic acid in the fat of the non-lactating gland is afforded by consideration of the studies of Popják *et al.*,[16] who investigated the synthesis of milk fatty acids from radioactive acetate in a lactating goat. Although all the saturated acids from butyric to palmitic were synthesized from acetate and showed significant specific activities, there was a pronounced fall in specific activity between palmitic acid and the $C_{18}$ acids, stearic and oleic, suggesting that the main bulk of these acids in milk fat probably had their origin in blood glycerides. Since secretion of milk, and hence rapid glyceride synthesis, had ceased in the animal studied in the present investigation, it is possible that chain lengthening of lower fatty acids had proceeded to a greater degree than normally occurs in the lactating gland; thus palmitic acid would accumulate, with a concomitant decrease in the amounts of lower homologues present.

The carotene content of the mammary-gland fats, particularly that of the lactating animal (55 μg./g.), is greater than that normally found in milk or colostrum fat. For butter fat, Morton *et al.*[4] found an average carotene content of 4·2 μg./g., McGillivray[17] found values of 6–11 μg./g. for New Zealand butter-fat, and Gillam & El Ridi[18] reported values of 0·8–1 μg./g. for butter fat and 2·4–6·2 μg./g. for colostrum fat. The carotene in the mammary gland may be stored as a caroteno-protein since it was extracted from the tissue much more readily with ethanol–ether than with acetone.

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# THE COLORIMETRIC DETERMINATION OF BENZENE HEXACHLORIDE IN INSECT TISSUES

By F. R. BRADBURY and H. STANDEN

Benzene hexachloride may be determined by hydrolysing it to trichlorobenzene, nitrating to trichlorodinitrobenzene and converting this into chlorodinitroresorcinol by hydrolysis with aqueous caustic soda. Under these conditions the main colour-producing compound is 6-chloro-2 : 4-dinitroresorcinol, but 2-chloro-4 : 6-dinitroresorcinol and 5-chloro-2 : 4-dinitroresorcinol may also contribute to the colour. Benzene hexachloride is separated from fats in insect tissues by percolating through a column packed with kieselguhr and a mixture of carbon tetrachloride and oleum.

In the course of work on the penetration of benzene hexachloride into insect tissues a colorimetric method for determining benzene hexachloride has been used. This process, which was developed by H. K. Southern and A. Williams, has been described in an earlier communication.[1] Since then the chemistry of the colour reaction has been studied, and the colour-producing compounds have been isolated and identified. Some improvement in the technique for extracting the benzene hexachloride from the insect have also been introduced.

## The chemical reactions and the compounds formed during the colorimetric analysis

In the production of a coloured compound from benzene hexachloride the first step is dehydrohalogenation to trichlorobenzene, followed by nitration and hydrolysis with alkali, when a yellow colour is obtained.

The dehydrohalogenation of benzene hexachloride was studied by Van der Linden.[2] All three isomeric trichlorobenzenes are formed. The proportions differ slightly according to the benzene hexachloride isomer and the dehydrohalogenating agent used, but in all cases studied by Van der Linden 1 : 2 : 4-trichlorobenzene was the major product formed.

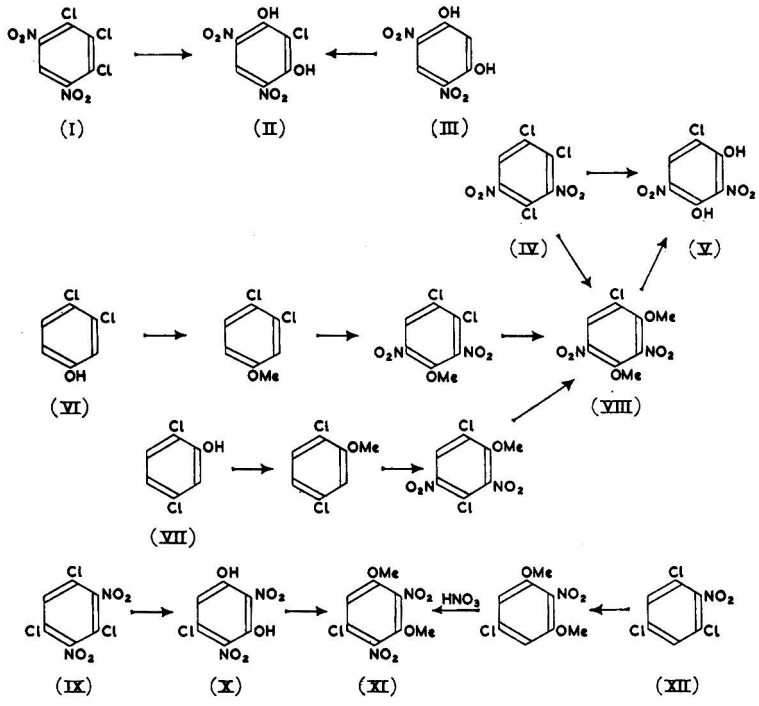
Nitration of trichlorobenzene was studied by Hüffer,[3] who found that heating with a mixture of equal portions of concentrated sulphuric acid and fuming nitric acid (the nitrating conditions used in the colorimetric reaction) converted each of the three trichlorobenzenes into the corresponding dinitro-derivatives. 1 : 2 : 3-Trichlorobenzene gave 1 : 2 : 3-trichloro-4 : 6-dinitrobenzene, 1 : 2 : 4-trichloro- gave 1 : 2 : 4-trichloro-3 : 5-dinitrobenzene, and 1 : 3 : 5-trichloro- gave 1 : 3 : 5-trichloro-2 : 4-dinitrobenzene.

The action of hot aqueous caustic soda on the trichlorodinitrobenzenes has not been studied previously. We found that the main product from each of the three trichlorodinitrobenzenes was a chlorodihydroxydinitrobenzene, two chlorine atoms having been replaced by hydroxyl groups. 1 : 2 : 3-Trichloro-4 : 6-dinitrobenzene (I) gave 2-chloro-4 : 6-dinitroresorcinol (II), which proved to be identical with a sample of 2-chloro-4 : 6-dinitroresorcinol prepared by chlorinating 4 : 6-dinitroresorcinol (III) by the method used by Kehrmann.[4]

1 : 2 : 4-Trichloro-3 : 5-dinitrobenzene (IV) gave 6-chloro-2 : 4-dinitroresorcinol (V). The structure of this compound was established by its synthesis from both 3 : 4-dichloro- (VI) and 2 : 5-dichloro-phenol (VII). In each case the phenol was methylated and the resulting anisole nitrated and treated with sodium methoxide. Both dichlorophenols gave the same chloro-dimethoxydinitrobenzene (VIII) which, on hydrolysis with aqueous caustic soda, gave 6-chloro-2 : 4-dinitroresorcinol (V) identical with the compound we obtained from 1 : 2 : 4-trichloro-3 : 5-dinitrobenzene.

1 : 3 : 5-Trichloro-2 : 4-dinitrobenzene (IX) gave 5-chloro-2 : 4-dinitroresorcinol (X) which, on methylation, gave 5-chloro-2 : 4-dinitroresorcinol dimethyl ether (XI), identical with a sample of this compound prepared from 1 : 3 : 5-trichloro-2-nitrobenzene (XII) by treatment with sodium methoxide and nitration as described by Hodgson & Batty.[5]

Van Rijn[6] recorded that sodium methoxide interacted with 1 : 3 : 5-trichloro-2 : 4-dinitrobenzene to give a mixture of the two possible isomers of chlorodinitroresorcinol dimethyl ethers, but there is no evidence in our work of the formation of 5-chloro-4 : 6-dinitroresorcinol by the action of aqueous caustic soda on 1 : 3 : 5-trichloro-2 : 4-dinitrobenzene. The yield of 5-chloro-2 : 4-dinitroresorcinol (52%; 2 g. of pure product from 4·5 g. of trichlorodinitrobenzene) and the absence of any other crystallizable product supports the view that the major product of the interaction of aqueous caustic soda with the compound is 5-chloro-2 : 4-dinitroresorcinol.




**Absorption spectrograms of the chlorodinitroresorcinols**

The absorption determinations were carried out on the 'Uvispek' spectrophotometer, with 1% solutions of the chlorodinitroresorcinol in 0·5% sodium carbonate solution (Fig. 1). The curves clearly show that the optical density of a solution of 6-chloro-2 : 4-dinitroresorcinol in 0·5% sodium carbonate solution is much greater than optical densities of corresponding solutions of the other two isomers at the wavelengths used for Spekker spectrophotometer readings of the end-point solutions in the colorimetric analysis of benzene hexachloride (No. 601 dark-blue Ilford filter).

Since 6-chloro-2 : 4-dinitroresorcinol is derived from 1 : 2 : 4-trichlorobenzene, which is the major product of the dehydrohalogenation of benzene hexachloride, it follows that the optical density of the final solution is due almost entirely to this isomer. Thus Van der Linden[2] gives

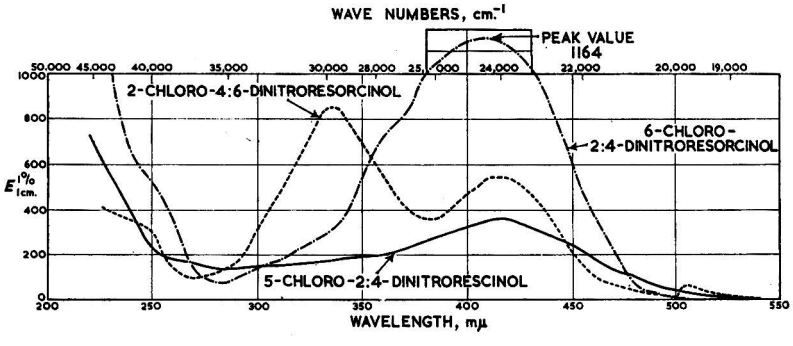



FIG. 1.—*Absorption spectrograms of the chlorodinitroresorcinols*

figures of 75·9% of 1 : 2 : 4-trichlorobenzene, 17·6% of 1 : 2 : 3-trichlorobenzene and 6·5% of 1 : 3 : 5-trichlorobenzene for the decomposition of α-benzene hexachloride by alcoholic sodium hydroxide solution at 80°. When the three resorcinols derived from these isomers were mixed in the proportion 76, 17 and 7% the optical density of the solution in the spectrophotometer with a M601 filter was 0·436, very close to that for 6-chloro-2 : 4-dinitroresorcinol (0·458) derived from 1 : 2 : 4-trichlorobenzene (see Table I).

**Table I**

| Compound | Optical density of 100 μg. in 10 ml. |
|---|---|
| 2-Chloro-4 : 6-dinitroresorcinol (I) | 0·362 |
| 6-Chloro-2 : 4-dinitroresorcinol (II) | 0·458 |
| 5-Chloro-2 : 4-dinitroresorcinol (III) | 0·340 |
| 76% of (II), 17% of (I), 7% of (III) | 0·436 (calc. 0·434) |

## The efficiency of the colorimetric analysis

The efficiency was determined by preparing calibration graphs for benzene hexachloride, trichlorobenzene, 1 : 2 : 4-trichloro-3 : 5-dinitrobenzene and 6-chloro-2 : 4-dinitroresorcinol. From these graphs the loss that takes place during each stage of the process was calculated.

Standard solutions of the four compounds were prepared with carbon tetrachloride as the solvent, except for the chlorodinitroresorcinol, when ether was used. Aliquots of these solutions were taken and subjected to the analytical procedure beginning at the appropriate stage. The benzene hexachloride analysis was started at the 'hydrolysis' stage, the trichlorobenzene at the second nitration stage, the trichlorodinitrobenzene at the stage at which the colour is produced by refluxing with aqueous sodium hydroxide solution, and the chlorodinitroresorcinol at the final stage, that is the extraction of the ethereal solution with 0·5% sodium carbonate solution.

All weights were converted to microgram equivalents of benzene hexachloride for the purpose of applying to calibration curves. These curves are reproduced in Fig. 2.

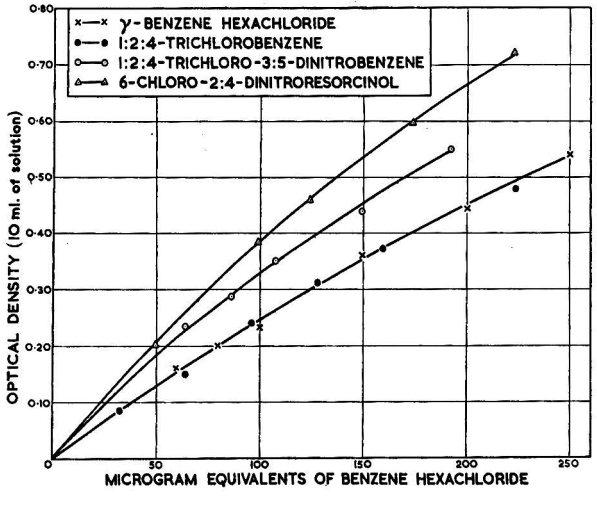



FIG. 2.—*Calibration curves*

It is evident from the curves that there is no loss of benzene hexachloride in the analytical process before the nitration of trichlorobenzene. Loss occurs during the nitration stage and, to a less degree, in the subsequent hydrolysis to chlorodinitroresorcinol. It can be estimated from the curves that some 40% of the benzene hexachloride is lost during the whole series of

reactions. The actual loss is probably less than this since the optical density of the mixed chlorodinitroresorcinols resulting from the treatment of benzene hexachloride is likely to be less than that of the 6-chloro-2 : 4-dinitro-compound (see Table I).

### Separation of benzene hexachloride from fats and other organic matter in insect tissues

In the earlier communication, Armstrong, Bradbury & Standen[1] separated benzene hexachloride from insect tissues by a digestion with concentrated nitric acid followed by steam distillation of the benzene hexachloride. This has now been replaced by a method based on that described for the separation of DDT from other organic matter by Armstrong, Bradbury & Britton.[7]

The insects were ground with anhydrous sodium sulphate in a pestle and mortar until finely subdivided and then extracted three times, with a total volume of 20 ml. of carbon tetrachloride, in a narrow cylindrical separating-funnel with a sintered-glass filter above the tap at the bottom. Each extraction occupied approximately four minutes, during which time the mixture was stirred occasionally with a glass rod. The carbon tetrachloride solution was then drawn through the sintered glass into a 50-ml. flask by means of suction from a water pump. The combined extracts were then subjected to a treatment to remove fats, etc.

A high-grade kieselguhr (Kensil P11) (3 g.) was mixed intimately with 30% oleum (2 ml.) in a mortar, and enough carbon tetrachloride added to form a stiff slurry. Carbon tetrachloride (5 ml.) was poured into a glass column (2 cm. diameter and 10 cm. long), closed at the lower end with a sintered-glass filter, and the mixture added, with light tamping, until a depth of about 4 cm. was attained.

When the excess of carbon tetrachloride had almost ceased to drain off, the extracts were poured carefully into the column and allowed to filter through into a 50-ml. flask. The column was washed through four times with 3–4 c.c. of carbon tetrachloride each time, and finally suction from the water pump was applied for half a minute to complete the process. The normal benzene hexachloride colorimetric analysis was then carried out, beginning with the first nitration.

New calibration graphs were prepared and the loss of benzene hexachloride was calculated to be approximately 11% compared with 35% ($\gamma$-isomer) and 52% ($\delta$-isomer) in the nitric acid and steam-distillation treatment previously used. The calibration graphs for $\gamma$- and $\delta$-isomers were identical.

For the removal of benzene hexachloride from external tissues a methanol wash was employed in the earlier work. It has now been found that a more efficient method is to use carbon tetrachloride. This is more convenient since the benzene hexachloride must be in carbon tetrachloride solution for the colorimetric analysis, and the inefficient operation of extracting benzene hexachloride from a dilute aqueous methanol solution with carbon tetrachloride, which is necessary after the methanol wash, is avoided. By this technique the loss of benzene hexachloride in that part of the process preceding the colorimetric analysis was reduced to approximately 4%, in comparison with a loss of approximately 30% when the methanol wash was employed.

Further experiments on insects exposed to benzene hexachloride showed that, as with the methanol wash, carbon tetrachloride wash removes benzene hexachloride from the outermost tissues only.

The calibration graphs for $\gamma$- and $\delta$-isomers were identical.

### Experimental

*Interaction of 1 : 2 : 3-trichloro-4 : 6-dinitrobenzene with aqueous sodium hydroxide*

The trichlorodinitrobenzene (5 g.) was boiled under reflux with 12% aqueous caustic soda (1 l.) for three hours. After the unchanged starting material was cooled and filtered off, the solution was acidified by the careful addition of concentrated sulphuric acid, with cooling. The product came out of solution and was filtered off and recrystallized, first from 30% acetic acid, and finally from light petroleum (b.p. 100–120°). Yellow needles, m.p. 183°, were obtained. [Found : C, 30·6 ; H, 1·3 ; N, 11·8 ; Cl, 15·4. $C_6HCl(NO_2)_2(OH)_2$ requires C, 30·7 ; H, 1·3 ; N, 11·9 ; Cl, 15·1%]. The compound was found by melting-point and mixed-melting-point determinations to be identical with a specimen of 2-chloro-4 : 6-dinitroresorcinol prepared by chlorinating a suspension of 4 : 6-dinitroresorcinol in ether by the method of Kehrmann.[4]

*Interaction of 1 : 2 : 4-trichloro-4 : 6-dinitrobenzene with aqueous sodium hydroxide*

The trichlorodinitrobenzene (5 g.) was refluxed with aqueous caustic soda solution as described above. The product was not precipitated on acidification, and was isolated by extraction with ether. After the extract was washed with a small quantity of water and then dried over anhydrous sodium sulphate, the ether was removed by distillation, leaving a dark-brown viscous liquid, which solidified on cooling and scratching with a glass rod. The solid was recrystallized from light petroleum (b.p. 60–80°), in the form of small yellow plates, m.p. 77° [Found: C, 30·8; H, 1·3; N, 11·8; Cl, 15·4. $C_6HCl(NO_2)_2(OH)_2$ requires C, 30·7; H, 1·3; N, 11·9; Cl, 15·1%].

*Conversion of 3 : 4-dichlorophenol and 2 : 5-dichlorophenol into 6-chloro-2 : 4-dinitroresorcinol*

The dichlorophenol was first methylated by shaking with dimethyl sulphate in excess of aqueous caustic soda; the product was an oil. This was separated off and nitrated by dissolving in fuming nitric acid at 15°. On being poured into water a solid was obtained, which was 3 : 4-dichloro-2 : 6-dinitroanisole from the 3 : 4-dichlorophenol, and 3 : 6-dichloro-2 : 4-dinitroanisole from the 2 : 5-dichlorophenol (Hollemann[5]).

The interaction of sodium methoxide (1 mole) with these compounds gave, in each instance, 6-chloro-2 : 4-dinitroresorcinol dimethyl ether, m.p. 68°. This was shown by melting-point and mixed-melting-point determinations to be identical with a specimen obtained by the action of sodium methoxide on 1 : 2 : 4-trichloro-3 : 5-dinitrobenzene (Hüffer[3]). This was treated with hot aqueous caustic soda in exactly the same way that 1 : 2 : 4-trichloro-3 : 5-dinitrobenzene had been treated, and the final product was the same. By virtue of its synthesis from 3 : 4- and 2 : 5-dichlorophenols it must therefore be a resorcinol, and hence is 6-chloro-2 : 4-dinitroresorcinol.

*Interaction of 1 : 3 : 5-trichloro-2 : 4-dinitrobenzene with aqueous sodium hydroxide*

The trichlorodinitrobenzene (5 g.) was boiled under reflux with 12% aqueous caustic soda by the method described above. No precipitate was formed on acidification, but some of the product crystallized out on being set aside overnight. The remainder was isolated by ether extraction by the procedure previously described. The product was recrystallized from light petroleum (b.p. 100–120°) and formed small orange-coloured needles, m.p. 117° [Found: C, 31·0; H, 1·3; N, 11·9; Cl, 15·1. $C_6HCl(NO_2)_2(OH)_2$ requires C, 30·7; H, 1·3; N, 11·9; Cl, 15·1%].

The structural formula was established by methylation, which was carried out by heating with aqueous caustic soda and excess of dimethyl sulphate. After all excess of dimethyl sulphate had been decomposed by addition of more caustic soda solution, the product was filtered off and recrystallized from alcohol m.p. 110°. It was proved by melting-point and mixed-melting-point determinations to be identical with a specimen of 5-chloro-2 : 4-dinitroresorcinol dimethyl ether, prepared according to the method used by Hodgson & Batty.[5] The original compound is therefore 5-chloro-2 : 4-dinitroresorcinol.

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# JOURNAL OF THE SCIENCE OF FOOD AND AGRICULTURE

## CONTENTS

PAGE

The chromogen method for determining the digestibility of dried grass by sheep .. .. .. .. .. 209
*By J. Davidson*

Physicochemical studies on the application of insecticides to sheep fleece. V.—The influence of carbon-chain length and halide ion of cationic wetting agents on their reaction with natural fleece .. .. .. .. .. 212
*By C. C. Addison and C. G. L. Furmidge*

The non-volatile organic acids of grass .. .. .. .. .. .. .. .. .. .. .. 221
*By A. C. Hulme and A. Richardson*

Connective tissue of meat. III.—Determination of collagen in tendon tissue by the hydroxyproline method .. 226
*By L. C. Baker, L. H. Lampitt and K. P. Brown*

A $^{14}C$ study of carbon dioxide fixation in the apple. I.—The distribution of incorporated $^{14}C$ in the detached McIntosh apple .. .. .. .. .. .. .. .. .. .. .. .. 231
*By N. Allentoff, W. R. Phillips and F. B. Johnston*

A $^{14}C$ study of carbon dioxide fixation in the apple. II.—Rates of carbon dioxide fixation in the detached McIntosh apple .. .. .. .. .. .. .. .. .. .. .. .. 234
*By N. Allentoff, W. R. Phillips and F. B. Johnston*

The isolation of an anthocyanin and chlorogenic acid from canned Victoria plums .. .. .. .. .. 239
*By T. L. Parkinson*

The component fatty acids of bovine mammary-gland fat .. .. .. .. .. .. .. .. 247
*By G. A. Garton*

The colorimetric determination of benzene hexachloride in insect tissues .. .. .. .. .. .. 252
*By F. R. Bradbury and H. Standen*

**Abstracts**



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