# PHENOLIC COMPOUNDS IN WHEAT FLOUR AND DOUGH

By H. P. C. GALLUS* and A. C. JENNINGS*

[Manuscript received October 30, 1970]

*Abstract*

Phenolic compounds covalently bound to macromolecules occur in wheat flour and are released by acid or alkaline hydrolysis. It is concluded that changes in phenolic compounds may partly account for the aging effect in flours.

## I. INTRODUCTION

Free phenolic compounds and derivatives of low molecular weight are present in rather small amounts in wheat flour and washed gluten (Gallus and Jennings 1971) and in whole ungerminated wheat grain (el-Basyouni and Towers 1964). Fausch, Kündig, and Neukom (1963) and Painter and Neukom (1968) have shown that a glycoprotein of wheat flour contains an esterified ferulic acid molecule and has rather unusual physical properties. Quinonoid compounds may interact with the sulphydryl group of cysteine and the $\epsilon$-amino group of lysine in proteins, causing changes in the physical and enzymic properties of the proteins (Mason 1955; Loomis and Battaile 1966; Pierpoint 1966, 1969*a*, 1969*b*; Anderson and Rowan 1967; Anderson 1968; Byck and Dawson 1968).

In this paper it is shown that wheat flour contains bound phenolic compounds which may affect the properties of dough.

## II. MATERIALS AND METHODS

Flour was milled from grain of *Triticum vulgare* cv. Gabo and cv. Mexico 120. Gluten was prepared by the procedure described by Kent-Jones and Amos (1957, p. 601).

### (*a*) *Methods of Hydrolysis*

*Hydrolysis 1.*—A suspension of Gabo flour (50 g) in water (200 ml) was extracted with two portions each of ether† and n-butanol. An equal volume of concentrated hydrochloric acid was added and the mixture left for 19 days at room temperature. The dark brown hydrolysate was neutralized to about pH 3 with 10N KOH (in an ice–water-bath) and extracted with ether and n-butanol, as before. The hydrolysate was adjusted to pH 8·5 with 10N KOH and re-extracted as before with ether and n-butanol.

*Hydrolysis 2.*—Gabo flour (50 g) was suspended in 6N HCl (200 ml) and left, with occasional mixing, for 64 hr at room temperature. The hydrolysate was extracted twice with ether and three times with n-butanol, adjusted to pH 10 with 10N KOH (in an ice–water-bath), then re-extracted with ether and n-butanol as before.

* Department of Agricultural Biochemistry and Soil Science, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, S.A. 5064.

† The ether used in these extractions was washed with dilute sodium hydroxide and distilled water immediately before use.

*Hydrolysis 3.*—Gabo flour (50 g) was suspended in 2N KOH (200 ml) for 64 hr at room temperature. It was necessary to add 400 ml distilled water to the tacky brown gel which formed for adequate dispersal before extraction with two portions of ether and three portions of n-butanol. The hydrolysate was adjusted to pH 2 with hydrochloric acid and re-extracted with ether and n-butanol, as above.

*Hydrolysis 4.*—Gluten was prepared from the flours of cv. Gabo and cv. Mexico 120 and thoroughly macerated with n-butanol in a mortar and pestle. The residue was washed with water and suspended in 6N HCl for 10 days at room temperature, with occasional mixing. The hydrolysate was neutralized to pH 3 with 10N KOH (in an ice–water-bath) and treated subsequently as for hydrolysis 1.

### (*b*) *Chromatographic Procedures*

The extracts were concentrated in a rotary evaporator and chromatographed on Whatman No. 1 paper with the following solvent systems (proportions by volume):

A: n-butanol–acetic acid–water, 4:1:2·2 (Cartwright and Roberts 1954);
B: n-butanol–acetone–water, 5:1:2 (Mitsuno 1953);
C: n-butanol saturated with concentrated ammonia (Lederer 1949);
D: 2, 6, or 10% acetic acid in water (Cartwright and Roberts 1954);
E: isopropanol–water, 4:1 (Smith 1960, p. 248);
F: the upper phase of benzene–formic acid–water, 1000:2:98 (Reio 1959);
G: the upper phase of benzene–acetic acid–water, 6:7:3 (Ibrahim and Towers 1960);
H: benzene–acetic acid–water, 127:72:3 (Smith 1960, p. 292);
J: n-propanol–water, 8:1 (Huennekens, Hanahan, and Uziel 1954);
K: ethanol–water, 8:1 (Huennekens, Hanahan, and Uziel 1954);
L: ethyl acetate–pyridine–water, 40:11:6 (Jermyn and Isherwood 1949).

The chromatograms were examined under ultraviolet light before and after exposure to ammonia vapour to detect fluorescent and absorbing zones, and then stained with the following reagents:

I: nigrosine, to detect polypeptides and proteins (Gallus and Jennings 1968);
II: ninhydrin, to detect amino acids and peptides (Stepka 1957; Mabry and Todd 1963);
III: sudan black, to detect lipids (Gurr 1960);
IV: silver nitrate, to detect sugars, phenolic, and other reducing compounds (Trevelyan, Proctor, and Harrison 1950; Benson *et al.* 1952);
V: ferric chloride–potassium ferricyanide, to detect phenolic and other reducing compounds (Kirby, Knowles, and White 1953);
VI: diazotized *p*-nitroaniline, to detect phenolic compounds (Swain 1953).

Some extracts contained considerable amounts of polypeptides, amino acids, sugars, lipids, and potassium chloride. The presence of potassium chloride made the chromatographic resolution of some components difficult. The relatively large amounts of lipid present in some extracts caused rather slow wetting of the material at the origin by aqueous solvents, thus affecting $R_F$ values by up to 10% in the developed chromatograms.

## III. Results

### (*a*) *Free Compounds of Low Molecular Weight*

Ether extracted a small amount of material from wheat flour whereas n-butanol extracted rather more (first extract, hydrolysis 1). Both extracts contained only lipid and small amounts of fluorescent material. After chromatography of both these extracts with solvent A, a small amount of fluorescent material coincided with the lipid zone and reagent V reacted with compounds in this zone to give two distinct zones.

The n-butanol extracts of both gluten preparations (first extracts, hydrolysis 4) contained much lipid, a little fluorescent material which reacted with reagent V, and rather small amounts of material which reacted with reagents I, II, and IV. The compounds which reacted with reagent IV had $R_F$ values corresponding to those of maltose and either glucose or fructose or both.

### (*b*) *Aging Effects*

When chromatograms of these extracts (i.e. first extracts, hydrolysis 4) were stored for a few days before staining, the zones which fluoresced originally had changed to zones which absorbed ultraviolet light and reacted much more intensely with reagent V. After storage for several months before staining, the zones which had changed from a fluorescent to an absorbing state showed further change to a highly fluorescent state. Chromatograms of the ether and n-butanol extracts of whole flour showed similar changes but at a much slower rate.

Portions of the n-butanol extracts of the gluten preparations (i.e. first extracts, hydrolysis 4) were applied to the origins of chromatograms. These were stored for several days until all the fluorescent material changed to a form which absorbed ultraviolet light. After development of the chromatograms with solvent B, small amounts of the lipid and material which absorbed ultraviolet light now remained at the origin and there was no reversion of any material to a form which fluoresced in ultraviolet light. The compounds which did migrate on these chromatograms showed the same mobilities and reaction with reagent V as they did in their fluorescent forms.

### (*c*) *Compounds Released by Hydrolysis*

Ether and n-butanol extracted much greater amounts of phenolic material from the acid and alkaline hydrolysates of the flour and gluten than from the untreated flour or gluten. This phenolic material reacted with reagents V and VI and either fluoresced in or absorbed ultraviolet light. It was detected, in varying amounts, in all the extracts of hydrolysates 1–4 and contained phenolic compounds, either free or apparently covalently bound to polypeptides or lipids. The chromatographic zones which did not absorb or fluoresce in ultraviolet light did not react with reagent V while some of the zones which reacted with reagent V also reacted with reagents IV and VI. However, reagent VI is much less sensitive than reagent V.

Phenolic compounds apparently covalently bound to polypeptides were extracted by n-butanol from acidic solutions. When freed of n-butanol and hydrochloric acid, this complex material would not redissolve in n-butanol or n-butanol saturated with water unless a small amount of ammonia was added.

Two fractions gave a greater number of zones of phenolic compounds on chromatography than the others and appeared to contain all of the free phenolic compounds detected in the various extracts. These were the ether extracts of the acid and acidified alkaline hydrolysates of the flour (hydrolysates 2 and 3). (After removal of the ether from the extract of the acid hydrolysates a small amount of material did not redissolve in ether but dissolved readily in ethanol.) Amino acids, lipid, saccharide, and polypeptide materials were not detected in the ether extract of the acidified alkaline hydrolysate.

Table 1 shows the $R_F$ values of the zones obtained after two-way chromatography of hydrolysates 2 and 3 in solvent systems F and D (these systems giving maximum resolution) and which reacted with reagents V or VI or fluoresced in or absorbed ultraviolet light. It is apparent that only a few of the compounds detected were present in both extracts.

TABLE 1

$R_F$ VALUES OF THE CHROMATOGRAPHIC ZONES WHICH REACTED WITH REAGENTS V OR VI, OR FLUORESCED IN OR ABSORBED ULTRAVIOLET LIGHT

Fractions chromatographed were either the first ether extract of the acid hydrolysate (hydrolysate 2) or the ether extract of the acidified alkaline hydrolysate (hydrolysate 3). Two-way chromatography carried out in solvent systems F (descending, first dimension) and D (ascending, second dimension), these solvent systems giving the maximum resolution

| $R_F$ | | Fluorescence in Ultraviolet Light | Reagent V | Reagent VI |
|---|---|---|---|---|
| Solvent F | Solvent D (2%) | | | |
| | | Hydrolysate 2 | | |
| 0 | 0 | Trace | Blue | Nil |
| 0 | 0·28 | Buff | Nil | Nil |
| 0 | 0·73 | Absorption | Faint blue | Nil |
| 0·07 | 0·54 | Light blue | Blue | Violet |
| 0·08 | 0·31 | Light blue | Blue | Violet |
| 0·14 | 0·53 | Light blue | Blue | Violet |
| 0·14 | 0·81 | Absorption | Faint blue | Nil |
| 0·15 | 0·32 | Light blue | Blue | Violet |
| 0·39 | 0·36 | Buff | Nil | Nil |
| 0·54 | 0·35 | Buff | Nil | Nil |
| 0·88 | 0 | (Lipid)* | Blue | Nil |
| | | Hydrolysate 3 | | |
| 0 | 0 | Buff | Blue | Pink |
| 0 | 0·16 | Blue | Blue | Pink |
| 0 | 0·52 | Blue | Blue | Pink |
| 0 | 0·77 | Absorption | Nil | Nil |
| 0·02 | 0·40 | Buff | Blue | Nil |
| 0·12 | 0·25 | Light blue | Blue | Pink† |
| 0·12 | 0·56 | Light blue | Blue | Pink† |
| 0·16 | 0·07 | Grey | Nil | Nil |
| 0·90 | 0 | Faint blue (lipid) | n.d. | Very faint pink |
| 0·94 | 0·69 | Absorption | n.d. | Nil |

* Absorption at centre of zone and fluorescence at circumference.

† Changing to violet.

The mobilities of the various compounds on two-dimensional chromatography did not agree with those of the phenolic compounds previously isolated from the ungerminated wheat grain by el-Basyouni and Towers (1964). Further, the known compounds which we tested gave quite different colours with reagent VI to those shown by the unknown compounds.

## IV. Discussion

### (*a*) *Free Phenolic Compounds*

The results confirm that only trace amounts of free phenolic compounds or phenolic derivatives of low molecular weight occur in wheat flour (el-Basyouni and Towers 1964; Gallus and Jennings 1971).

### (*b*) *Bound Phenolic Compounds*

The extraction of much larger amounts of different phenolic compounds and derivatives from the hydrolysates of wheat flour and gluten shows that this type of compound occurs mainly bound covalently to other molecules. Our failure to detect free ferulic acid in the hydrolysates may have been due to either the ferulic acid remaining bound to sugar (Fausch, Kündig, and Neukom 1963; Painter and Neukom 1968) (or to other residues), thus affecting its mobility during chromatography, or to the occurrence of much lower levels of it relative to those of the other phenolic compounds present.

We failed to detect any of the phenolic acids found in small amounts by el-Basyouni and Towers (1964) in acid and alkaline hydrolysates of the ethanol extracts of ungerminated whole grain. However, they did not determine whether these were localized in any particular morphological fraction.

Some zones on the chromatograms gave reactions for both phenolic and polypeptide material. The failure of any of several solvent systems to separate this material into separate zones suggests that the phenolic residues are covalently bound to the polypeptides. The solubility properties of this material in acidified, neutral, and alkaline n-butanol suggests that the polypeptide material contained cysteic acid or aspartic and glutamic acid residues or all of these in addition to the basic amino acid residues necessary for the reaction with nigrosine. Although most of the aspartic and glutamic acid residues in flour proteins occur as their amides, hydrolysis of the amide groups of asparagine and glutamine occurs readily in strong acid and alkaline solutions.

### (*c*) *Phenol–Protein Interactions*

As far as we know there are no reports in the literature of proteins containing phenolic compounds attached directly to amino acid residues as part of their native structure. Phenolic compounds in their quinonoid form readily form addition products with the sulphydryl group of cysteine, the $\epsilon$-amino group of lysine, and the $\alpha$-amino group of amino acids (Mason 1955; Loomis and Battaile 1966; Pierpoint 1966, 1969*a*, 1969*b*; Byck and Dawson 1968). Thus it is probable that the phenolic compounds, either free or bound to other molecules in naturally occurring compounds, interacted in a random manner with these reactive amino acid groups in the wheat proteins at some time after protein synthesis had occurred. During the final stages of maturation of the grain, dehydration of the endosperm occurs with disruption of the functional organized structure of the cell and, presumably, of the metabolic control systems, thus allowing these addition products to form (Jennings and Morton 1963; Jennings *et al.* 1968). It is also possible that phenolic compounds, released by hydrolysis, interacted with the reactive amino acid groups, although it is unlikely

that this would occur to any extent in strongly acid solutions (Mason 1955; Byck and Dawson 1968; Pierpoint 1969*a*, 1969*b*).

The recovery of reactive phenolic compounds after acidification of the alkaline hydrolysates suggests that they were protected against oxidation by alkali-stable, acid-labile bonds.

We conclude also that the phenolic compounds are bound by different types of bonds into more than one type of molecule since significant amounts are recovered after the acid and alkaline hydrolysates are made alkaline or acidic, respectively. The differences in solubility noted also support this.

### (*d*) *Phenolic Compounds and Dough Properties*

Our failure to positively identify any of the phenolic compounds in the various extracts may have been due to the incomplete removal of saccharide or other molecules during hydrolysis. While the $R_F$ values of several zones corresponded to those of known compounds in particular solvent systems, they differed in others. Nevertheless it is apparent that significant amounts of reactive phenolic compounds occur in wheat flour and their possible effects on the rheological and other properties of flour and dough must be considered.

Qualitatively the same compounds were isolated from the glutens of cv. Gabo and cv. Mexico 120 in about the same apparent amounts. Thus it is unlikely that varietal differences in baking quality can be attributed to differences in the content and nature of phenolic compounds.

The naturally occurring glycoprotein containing ferulic acid readily forms a gel under appropriate conditions (Painter and Neukom 1968). Similar interactions leading to gel formation could conceivably occur between appropriate phenolic compounds randomly attached to protein molecules. The possibility also exists for crosslinking of polypeptide chains through phenolic compounds (Mason 1955; Loomis and Battaile 1966). In addition, the attachment of a phenolic compound to a polypeptide may cause perturbation of the conformation of the polypeptide and changes in its physical and other properties (Anderson 1968; Pierpoint 1969*b*).

The aeration of doughs during mixing would promote enzymatic or non-enzymatic oxidation of phenolic compounds to their quinonoid forms and thus facilitate their reaction with suitable amino acid residues. Thus it is necessary to consider the possible involvement of phenolic compounds in any study of the effects on the properties of doughs of oxidizing or reducing agents, and of compounds such as *N*-ethylmaleimide which react specifically with sulphydryl groups.

We suggest that the baking quality of flours may be affected by interactions between phenolic compounds and reactive groups in protein. However, there is as yet no evidence of the magnitude of this effect.

### (*e*) *Aging of Flour*

The changes in phenolic compounds observed on unstained chromatograms may also occur in wheat flour during aging and partly account for the observed changes in the properties of flour during storage (Kent-Jones and Amos 1957, p. 317; Pratt 1964).

## V. Acknowledgments

The authors wish to thank Professor D. J. D. Nicholas for his interest in this work. Grateful acknowledgment is made of financial support from the Wheat Industry Research Fund.

## VI. References

Anderson, J. W. (1968).—*Phytochemistry* **7**, 1973.
Anderson, J. W., and Rowan, K. S. (1967).—*Phytochemistry* **6**, 1047.
el-Basyouni, S., and Towers, G. H. N. (1964).—*Can. J. Biochem.* **42**, 203.
Benson, A. A., et al. (1952).—*J. biol. Chem.* **196**, 703.
Byck, J. S., and Dawson, C. R. (1968).—*Analyt. Biochem.* **25**, 123.
Cartwright, R. A., and Roberts, E. A. H. (1954).—*Chemy Ind.* p. 1389.
Fausch, H., Kündig, W., and Neukom, H. (1963).—*Nature, Lond.* **199**, 287.
Gallus, H. P. C., and Jennings, A. C. (1968).—*Aust. J. biol. Sci.* **21**, 1077.
Gallus, H. P. C., and Jennings, A. C. (1971).—*Aust. J. biol. Sci.* **24**, 825–8.
Gurr, E. (1960).—In "Encyclopaedia of Microscopic Stains". (Ed. E. Gurr.) p. 376. (Leonard Hill: London.)
Huennekens, F. M., Hanahan, D. J., and Uziel, M. (1954).—*J. biol. Chem.* **206**, 443.
Ibrahim, R. K., and Towers, G. H. N. (1960).—*Archs Biochem. Biophys.* **87**, 125.
Jennings, A. C., and Morton, R. K. (1963).—*Aust. J. biol. Sci.* **16**, 318.
Jennings, A. C., Pusztai, A., Synge, R. L. M., and Watt, W. B. (1968).—*J. Sci. Fd Agric.* **19**, 203.
Jermyn, M. A., and Isherwood, F. A. (1949).—*Biochem. J.* **44**, 402.
Kent-Jones, D. W., and Amos, A. J. (1957).—In "Modern Cereal Chemistry". 5th Edn. (Eds. D. W. Kent-Jones and A. J. Amos.) (Northern Publ. Co.: Liverpool.)
Kirby, K. S., Knowles, E. J., and White, T. (1953).—*J. Soc. Leath. Trades Chem.* **37**, 283.
Lederer, M. (1949).—*Aust. J. Sci.* **11**, 208.
Loomis, W. D., and Battaile, J. (1966).—*Phytochemistry* **5**, 423.
Mabry, C. C., and Todd, W. S. (1963).—*J. Lab. clin. Med.* **61**, 146.
Mason, H. S. (1955).—*Adv. Enzymology* **16**, 105.
Mitsuno, M. (1953).—*Pharm. Bull., Tokyo* **1**, 170.
Painter, T. J., and Neukom, H. (1968).—*Biochim. biophys. Acta* **158**, 363.
Pierpoint, W. S. (1966).—*Biochem. J.* **98**, 567.
Pierpoint, W. S. (1969*a*).—*Biochem. J.* **112**, 609.
Pierpoint, W. S. (1969*b*).—*Biochem. J.* **112**, 619.
Pratt, D. B. (1964).—In "Wheat Chemistry and Technology". (Ed. I. Hlynka.) p. 195. (American Association of Cereal Chemists: St. Paul, Minn.)
Reio, L. (1959).—*Chromat. Rev.* **1**, 39.
Smith, I. (1960).—In "Chromatographic and Electrophoretic Techniques". 2nd Edn. (Ed. I. Smith.) Vol. 1. (William Heinemann Medical Books Ltd.: London; Interscience: New York.)
Stepka, W. (1957).—In "Methods in Enzymology". (Eds. S. P. Colowick and N. O. Kaplan.) Vol. 3. p. 504. (Academic Press Inc.: New York.)
Swain, T. (1953).—*Biochem. J.* **53**, 200.
Trevelyan, W. E., Proctor, D. P., and Harrison, J. S. (1950).—*Nature, Lond.* **166**, 444.