# DNA COMPARISONS AMONG BARLEY, OATS, RYE, AND WHEAT[1]

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OF all groups of plants, none can match the cereal grains in importance to man. Their origins in cultivation have been traced back some 9,000 years to the beginnings of agriculture (HELBAEK 1960). Extensive cytogenetic studies have been undertaken with this group of plants from which their taxonomic and supposed evolutionary relationships may be deduced (BELL 1965; RILEY 1965). Some of the relationships in the family to which the cultivated cereals belong are diagrammed in Figure 1 which includes only the relevant genera. The grass family, Gramineae, contains a very large number of polyploid forms. STEBBINS (1956b) has calculated that nearly 70% of grass species are polyploid, a value which is twice the average of flowering plants as a whole. In addition there has been a striking development of allopolyploid groups including the three major crop plants: barley, oats and wheat. STEBBIN's (1956a,b) suggestions for a revision of the scheme presented in Figure 1 include the proposition that the tribe Triticeae be considered as a single genus. This change was proposed on the basis of cytological evidence and the uniquely high number of bigeneric hybrids. BOWDEN (1959) proposed on legal criteria that Triticum and Aegilops be considered as members of a single genus instead of separate genera in the subtribe Triticinae. They were so incorporated by MORRIS and SEARS (1967).

The present investigation was initiated to compare DNA base sequences within the Gramineae. DNA comparisons can yield quantitative data on relatedness and divergence among organisms (SCHILDKRAUT *et al.* 1962; MCCARTHY and BOLTON 1963; HOYER, MCCARTHY and BOLTON 1964; LAIRD and MCCARTHY 1968). BENDICH and BOLTON (1967) have recently demonstrated the applicability of these same methods to plant material. The basis of this approach depends upon the accumulation of heritable changes in the genes of organisms manifested in the base sequence of DNA. Such changes occur in the sequential order of bases in the polynucleotide structure of DNA and in the number of similar regions of DNA created by gene duplication. By separating the two DNA strands of one organism and recombining them with separated DNA strands of another organism, a heteroduplex or hybrid DNA is produced. The quantity of hybrid DNA is easily measurable when one of the two DNAs is radiolabeled.

It is generally recognized that the thermal stability of a DNA/DNA or RNA/

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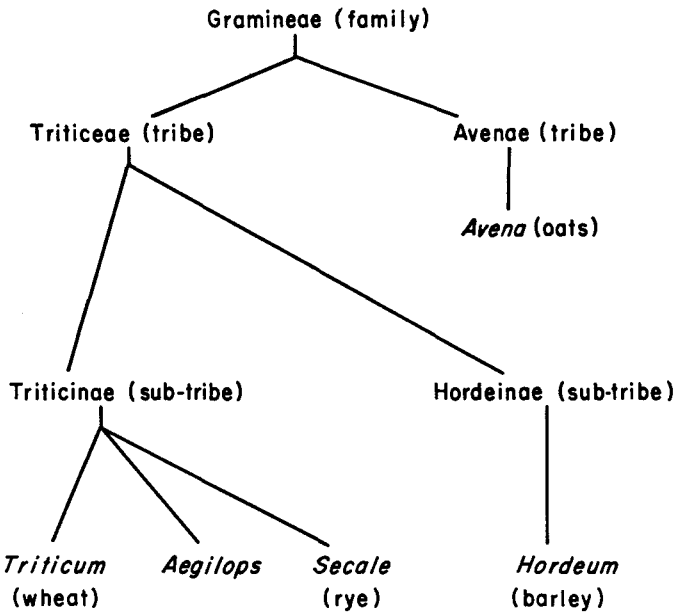


FIGURE 1.—Taxonomic relationships among some members of the grass family. Information taken from BELL (1965).

DNA hybrid is a function of the complementarity in base sequence of its two component strands (BAUTZ and BAUTZ 1964; KOTAKA and BALDWIN 1964; LAIRD and MCCARTHY 1968; MOORE and MCCARTHY 1968). The temperature at which 50% of a hybrid is dissociated is called the mean thermal dissociation temperature ($T_m$) and the difference in $T_m$ of homologous and heterologous hybrids is a measure of base sequence divergence between these DNAs. A $T_m$ difference of about 1°C is easily measurable and corresponds to about 1.5% base substitutions between two DNAs (LAIRD, MCCONAUGHY and MCCARTHY 1969).

Comparisons were made of the DNA of barley, oats, rye, and wheat, and also of some putative genome donors of hexaploid wheat (BENDICH and MCCARTHY 1970). The results of the present studies are considered in terms of supposed evolutionary relationships among these cereal grains.

## MATERIALS AND METHODS

Seeds were kindly donated as designated. Tetraploid wheat (*Triticum durum* Desf., cv. Leeds = *T. turgidum* var. *durum*) and rye (*Secale cereale* L., cv. Antelope), K. L. LEBSOCK (USDA, Fargo, N. Dakota). Hexaploid wheat (*Triticum aestivum* L. em. Thell. cv. Seneca), H. N. LEFEVER (Ohio Agricultural Research and Development Center, Wooster, Ohio). Barley (*Hordeum vulgare* L., cv. Himalaya), A. KLEINHOFS (Washington State University). Oats (*Avena sativa* L., cv. Victory), R. E. CLELAND (University of Washington).

Hexaploid wheat DNA was used in all experiments except those summarized in Table 4 where both hexaploid and tetraploid wheat DNAs were used.

Carbon-14 labeled *Bacillus subtilis* DNA was the gift of M. CHILTON.

*Growth of plants and extraction of DNA*: For extraction of unlabeled DNA, seeds were

germinated and grown in tap water in trays of Pearlite. After 1–2 weeks the seedlings reached a height of 8–15 cm and the shoots were cut off about 2–5 cm above the surface. The shoots were cut into approximately 3 cm segments, wrapped in aluminum foil and frozen at —80°C. Plants labeled with tritium labeled thymidine were either processed prior to freezing or stored frozen. The frozen plant material was pulverized to a powder either by grinding by hand with a dry-ice chilled mortar and pestle or in a chilled mill (Rollmix blender, Universal Distributors, Culver City, California) designed to make flour from grain. The first method was used for small quantities of tissue (up to 10 grams), as when labeled plants were processed. The second method was more convenient when working with up to 300 grams of frozen tissue. The goal was to break up the tissue as much as possible without permitting it to thaw and allow enzymatic degradation of the DNA. Small fragments of dry ice were added to the frozen tissue in the mill just prior to pulverization.

DNA was extracted from fresh or frozen powdered tissue as described earlier (BENDICH and BOLTON 1967). The tissue (shoots or whole plants minus the seed) was ground at room temperature with a mortar and pestle with sufficient extraction medium (0.5 M NaCl, 0.01 M sodium ethylenediaminetetraacetate pH 8, 1% sodium lauryl sulfate) to yield a thick paste. The volume used was 3–7 ml per 10 grams of tissue and was kept to a minimum to facilitate subsequent ethanol precipitation of the DNA. In place of the NaCl, citrate buffer used in the earlier procedure, 0.015 M NaCl, 0.01 M Tris pH 8 was substituted. Seeds were placed in a sterile beaker and soaked for 5–10 min sequentially in each of the following solutions: 3% Roccal (a cationic disinfectant), 10% O-syl (an anionic disinfectant), and 1–1.5% sodium hypochlorite (commercial bleach). All subsequent steps were carried out in a tissue culture hood to minimize bacterial contamination. The seeds were rinsed three times with sterile water and transferred to a sterile Petri dish containing filter paper and sufficient water to keep the seeds moist. Fifteen to 35 germinating seeds (2–4 days after planting) were transferred to a dish 15 cm high and 8 cm in diameter fitted with a lid as in a Petri plate. Two hundred to 350 $\mu$c $^3$H-thymidine (1 mc/0.015–0.036 mg; purchased from New England Nuclear Co.) dissolved in 3–5 ml sterile water were added to the plants and additional water was added during the labeling period of 5–7 days when necessary.

After extensive investigation on the effects of bacterial contamination, procedures were developed for axenic labeling of plant nucleic acids. The labeled plant DNA used in this work did not contain detectable bacterial DNA (BENDICH 1969).

*DNA-filter preparation*: DNA was denatured at 30–50 $\mu$g/ml in 0.01 × SSC (SSC = 0.15 M NaCl, 0.015 M trisodium citrate) at 95–100°C for 5 min and quickly transferred to 4 volumes of 0.01 × SSC in an ice bath. Twenty × SSC was added to give a final concentration of 4 × SSC. The DNA solution at 10 $\mu$g/ml was passed through a nitrocellulose filter (SCHLEICHER and SCHUELL, type B-6); the filter was washed with 4 × SSC before and after immobilization of the DNA (GILLESPIE and SPIEGELMAN 1965). The filter was then soaked for at least 20 min in DENHARDT's (1966) preincubation mixture and the excess liquid removed. Filters of 6.3 or 7.2 mm diameter were cut from the large filters (110 mm or 72 mm) with a paper punch or No. 3 cork borer and stored at least 2 hr at room temperature before baking at 60°C overnight. The quantity of DNA bound by the large filter was determined by measuring the absorbance at 260 m$\mu$ before and after passing the solution through the filter. Filters were stored at 4°C in sterile Petri dishes until used.

*DNA/DNA duplex formation using filter-bound DNA*: DNA/DNA duplexes were formed by incubation for 17 hr in 0.2 ml of SSC or 2 × SSC in 10 × 40 mm vials with one or more DNA-filters per vial. About 0.2 ml mineral oil was added to the vials to retard evaporation, and the vials were covered. One $\mu$g radiolabeled DNA which was sheared at 680–1020 atm (10–15,000 psi) in a French pressure cell (WARING and BRITTEN 1966) and then heat denatured was used in all reactions, but the number of DNA filters (each with 7–9 $\mu$g plant DNA) per vial varied from 1–5. The total DNA on all filters used per reaction is stated as "x $\mu$g filter-bound DNA." Guinea pig DNA-filters were used to monitor nonspecific hybridization when indicated, and were present in the reaction vials along with plant DNA-filters during the cereal grain direct binding reactions. Guinea pig DNA-filters were incubated in separate vials for the thermal

stability measurements. The incubation temperature varied from 37°C–65°C as indicated and various quantities of formamide were present. After incubation, the filters were washed for 5 min twice in 5 ml reaction buffer at the reaction temperature. When formamide was a constituent of the reaction mixture, the filters were subsequently washed for 3–5 min at room temperature in the reaction mixture lacking formamide. The filters were then blotted, skewered on pins, dried and counted.

*DNA-filter thermal stability profiles*: Washed DNA-filters containing duplexed labeled DNA were skewered on pins so that they did not touch each other and were held at the temperature of incubation in 5 ml SSC for 5 min. The pins with the filters were transferred to another 5 ml SSC using a paper clip holding a looped portion of the pin and were held for 5 min at a higher temperature. Up to 5 filters were used on one pin and filters from duplicate incubations were sometimes mounted on the same pin. Twenty μg beef serum albumin (BSA) was added to the cooled tubes whose contents were then precipitated with 5% TCA (trichloroacetic acid), filtered (Bac-T-Flex membrane filters, type B-6, SCHLEICHER and SCHUELL), dried and counted. The cumulative percent of total radioactivity eluted at all temperatures was plotted *vs.* temperature to give a thermal stability profile and the $T_m$ was designated as that temperature at which 50% of the radioactivity was eluted. Radioactivity remaining on the filters after the maximum temperature was reached (99–101°C) was always less than 2% of that reacting. These counts were not included in the calculations. The difference in $T_m$ between the homologous and heterologous duplexes ($\Delta T_m$) is reported only for mean thermal dissociation temperatures measured simultaneously.

*DNA reassociation in solution*: Sheared DNA was incubated at various concentrations in the salt and formamide concentrations indicated. For any given $C_0t$ curve (kinetics of DNA strand reassociation plotted as percent reassociation *vs.* the initial concentration × time; BRITTEN and KOHNE 1968), a stock solution of DNA was prepared and aliquots from this stock, or a dilution thereof, were placed in disposable, calibrated micropipets, the ends of which were then sealed. $C_0t$ curves were always determined with at least three concentrations of DNA incubated to values of $C_0t$ such that the points overlapped. Sealed micropipets (calibrated from 5–100 μl) containing from 3–135 μl of DNA were heated to 75°C or 85°C (well above the $T_m$ in the solvents used) for 5–8 min and transferred immediately to a 37°C or 40°C water bath. The samples were transferred to 2 ml of chilled phosphate buffer to stop reassociation. This material was stored at —20°C until fractionated on hydroxyapatite columns.

*Hydroxyapatite fractionation*: Single and double stranded DNA may be separated by use of hydroxyapatite (HA) (BERNARDI 1965). The procedures used here were adapted from previous work (MIYAZAWA and THOMAS 1965; BRITTEN and KOHNE 1968). HA (hydroxylapatite HT, purchased from BioRad) was equilibrated three times with 0.12 M Na phosphate buffer, pH 6.8 (PB) and resuspended in 1 ml PB per ml settled HA. One, 1.5, or 2 ml of this 1 to 1 suspension was placed into Pasteur pipets plugged with Pyrex glass wool and washed twice with 2.5 ml of PB. A plastic animal cage having holes fitted with stoppers with holes for the Pasteur pipets served as a water bath containing 11 such columns which were maintained at 60°C by an immersion heater-circulator. The ability of 0.7 ml packed HA in these columns to retain sheared native $^3$H-wheat DNA was unaffected by the presence of 180 μg sheared native rat DNA. Amounts of DNA up to 50 μg were assayed with this column. Samples were applied to the columns in 2 ml 0.12 M PB at 60°C and the columns were washed twice with 2.5 ml 0.12 M PB: DNA in this 7 ml effluent was not reassociated. Two 2 ml 0.5 M PB effluents were used to measure reassociated DNA. DNA sheared at 10–15,000 psi was used exclusively since higher molecular weight DNA was found to fractionate poorly on HA. BSA was added to the two chilled fractions and the 0.12 M PB fraction was made 5% in TCA, while the 0.5 M PB was made 10% in TCA. The precipitates were trapped on membrane filters, which were subsequently dried and counted. Percent reassociated DNA in a sample was calculated as follows:

$$\% \text{ Reassociated DNA} = \frac{B}{A + B} \times 100$$

A and B are the cpm in 0.12 M and 0.5 M PB fractions, respectively.

In citrate concentrations above $4 \times 10^{-3}$ M native DNA passes through HA columns, whereas below this concentration HA binds native DNA (BENDICH 1969; BERNARDI 1969). This fact is important since saline-citrate buffer is often used in DNA reassociation reactions.

*Radioactivity Measurements:* Samples were counted in a Packard Tri-Carb scintillation counter in Liquifluor (purchased from Nuclear Chicago) diluted with toluene. The efficiency of counting tritium hybridized on DNA-filters proved to be 50% higher than for $^3$H-labeled DNA TCA-precipitated in the presence of 20$\mu$g BSA carrier. All experiments were performed with one preparation of $^3$H-DNA from each biotype.

## RESULTS

*Base composition of cereal grain DNA*: The base composition of the DNA is a crude but useful parameter for species characterization. The DNA base compositions of the four principal cereal grains were determined from their buoyant densities and thermal denaturation temperatures.

Table 1 lists the buoyant density in CsCl along with the base compositions calculated from these measurements. The density of 1.701 for barley DNA is in close agreement with the 1.702 reported by LEDOUX and HUART (1968). The 1.702 value for wheat DNA density agrees exactly with the density reported by SCHILDKRAUT, MARMUR and DOTY (1962) for wheat-germ DNA, but differs from the 1.707 value reported for wheat root DNA (HOTTA, BASSEL and STERN 1965). CHEN and OSBORNE (1970) found densities of 1.702–1.706 for both germinated and ungerminated wheat embryo DNAs. There appears to be a small but significant difference among this group of DNAs. The absorbance bands were symmetrical and no secondary bands were detected.

The density of DNA in CsCl is decreased by substitution of 5-methylcytosine for cytosine (KIRK 1967). The mole percent guanine plus cytosine (G+C) of wheat DNA in Table 1 is therefore underestimated due to the substantial amount of 5-methylcytosine present in wheat-germ DNA whose chemically determined base composition is 47% G+C (SCHILDKRAUT, MARMUR and DOTY 1962). As-

TABLE 1

*Buoyant density in CsCl and thermal denaturation temperature of some cereal grain DNAs*

| Source of DNA | Density | $T_m$ (°C) | Mole % G+C calculated from density | Mole % G+C calculated from $T_m$ |
|---|---|---|---|---|
| Barley | 1.701 | 85.2 | 42 | 39 |
| Oats | 1.700 | ... | 41 | .. |
| Rye | 1.702 | 86.4 | 43 | 42 |
| Wheat | 1.702 | 85.2 | 43 | 39 |

One to two $\mu$g of the sample DNA and 1 $\mu$g *M. xanthus* DNA (density 1.727 g/cc) were centrifuged in CsCl with 0.1 × SSC at 42,040 rpm and 24°C for 20 hr in a Spinco Model E ultracentrifuge. The buoyant density and percent G+C of the DNAs were determined as described by SCHILDKRAUT, MARMUR and DOTY (1962). Sheared DNA was heated in 1 × SSC at a continuous rate in a Beckman DU spectrophotometer equipped with a Gilford automatic temperature and absorbance recorder. The initial $OD_{260}$ and percent hyperchromic effect was 1.8 and 33 for barley, 2.1 and 31 for rye, and 1.2 and 30 for hexaploid wheat. Percent G+C was calculated from the $T_m$ as described by MARMUR and DOTY (1962).

suming the other grain DNAs contain 5-methylcytosine, their percent G+C listed would also be underestimated.

The thermal denaturation temperature ($T_m$) of 85.2°C for wheat leaf DNA in Table 1 is markedly lower than the 88.5°C reported for wheat-germ DNA by MARMUR and DOTY (1962). CHEN and OSBORNE (1970) reported $T_m$ values of 86.5–87°C for ungerminated, and 84.5°C for germinated wheat embryo DNAs, and presented evidence indicating a modification of embryo DNA upon germination.

*Parameters of DNA/DNA duplex formation*: The formation of DNA/DNA duplexes involves the incubation of single stranded radiolabeled DNA in solution with unlabeled single stranded DNA immobilized on nitrocellulose filters. The product is a duplex structure held together by complementary base pairing approaching that in native double stranded DNA. The nature of the duplex depends on the conditions of formation since a wide variety of structures may be formed among the DNA molecules representing various members of gene families containing partially redundant base sequences. Detailed analyses of the specificity of the DNA/DNA duplex forming and RNA/DNA hybridization reaction have shown that the more stringent the conditions under which duplexes are formed, the greater is their average stability and fidelity of base pairing (MCCARTHY and MCCONAUGHY 1968; CHURCH and MCCARTHY 1968). Stringency varies inversely with salt concentration and directly with temperature and formamide concentration. The effects of some of these conditions on the interaction of plant DNAs will be considered before presentation of results dealing with base sequence relationships among various plant genomes.

Table 2 shows the effects on plant DNA comparisons of varying the temperature during duplex formation. As the temperature is increased, the mean thermal stability ($T_m$) of both the homologous (barley-barley) and heterologous (barley-rye) DNA duplexes increases. The increase in $T_m$ of the heterologous hybrid is greater than that of the homologous hybrid, and consequently the difference ($\Delta T_m$) becomes smaller as the temperature increases. Figure 2 presents thermal stability data in the form of "melting curves." The curves are steeper and closer

TABLE 2

*Effect of incubation temperature on DNA/DNA duplex formation*

| Temperature (°C) | $T_m$(°C) Barley | $T_m$(°C) Rye | $\Delta T_m$(°C) | Rye as percent of homologous bindings* |
|---|---|---|---|---|
| 55 | 72.2 | 66.3 | 5.9 | 126 |
| 60 | 72.7 | 69.1 | 3.6 | 65 |
| 65 | 74.3 | 71.2 | 3.1 | 51 |

Twenty-six μg barley, 22 μg rye or 27 μg rye (at 60°C) DNA bound to filters were incubated for 17 hr in 0.2 ml SSC at the indicated temperature with 1 μg $^3$H-labeled barley DNA (3200 cpm). Thermal stability profiles and $T_m$ values were determined (see Figures 2 and 3a). Of the input $^3$H-DNA, 28%, 40%, and 30% was eluted from the homologous barley DNA-filters at 55°C, 60°C, and 65°C, respectively.

* Total cpm eluted from rye DNA-filters are expressed as percent of total cpm eluted from barley DNA-filters (set at 100%).

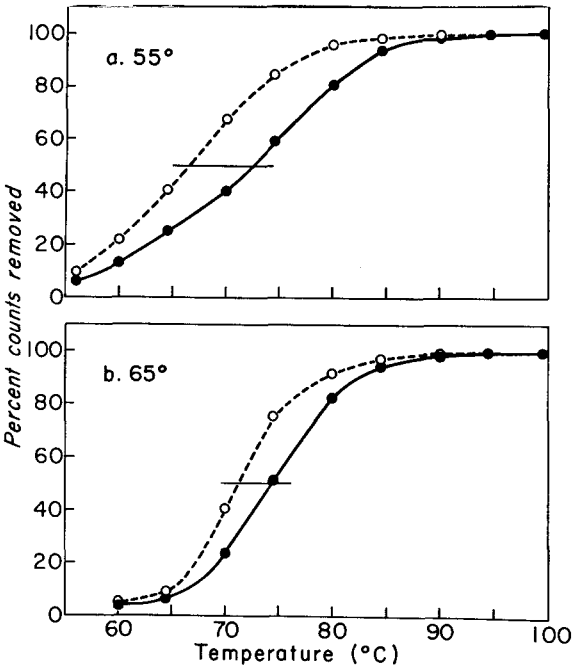


FIGURE 2.—Thermal stability profiles of DNA/DNA duplexes formed at 55°C and 65°C. $^3$H-labeled barley DNA was incubated with barley (filled circles) or rye (open circles) DNA-filters at (a) 55°C or (b) 65°C. Details are given in Table 2.

together for duplexes formed at 65°C than for those formed at 55°C. The relative binding of $^3$H-barley DNA to rye DNA-filters compared with $^3$H-barley DNA to barley DNA-filters decreases at higher temperature. The amount of $^3$H-barley DNA bound to rye DNA at low temperature is high, but the fidelity in base pairing is low, as judged by the mean thermal stability. A similar result was observed earlier for vertebrate DNAs (MARTIN and HOYER 1966). The value of $\Delta T_m$ may be used to calculate the fraction of base pair substitution between two DNAs (LAIRD, MCCONAUGHY and MCCARTHY 1969), and is therefore a valuable parameter of genetic relatedness.

The ratio of filter-bound DNA to labeled DNA also affects the $T_m$ and $\Delta T_m$ values (Table 3). Increased $T_m$ values were also obtained for wheat-wheat and wheat-rye duplexes with lower amounts of filter-bound DNA. This effect has been previously noted with rabbit ribosomal RNA-DNA hybrids (MOORE and MCCARTHY 1968). A likely explanation of this phenomenon is that the DNA sites on the filter which form the most stable base pairs with labeled DNA are preferentially filled. When there are a larger number of available sites, less well-paired structures are formed.

The addition of formamide to aqueous solutions decreases the thermal stability of DNA (BONNER, KUNG and BEKHOR 1967; MCCONAUGHY, LAIRD and MCCARTHY 1969). By adjusting the formamide concentration in DNA solutions, optimal conditions of reaction may be obtained at low temperatures. This offers

TABLE 3

*Effect of the ratio of filter-bound to labeled DNA on DNA/DNA duplex formation*

| μg Filter-bound DNA | $T_m$(°C) Rye | Wheat | $\Delta T_m$(°C) | Wheat as percent of homologous binding* |
|---|---|---|---|---|
| 27 | 74.9 | 70.8 | 4.1 | 54 |
| 7 | 76.9 | 74.0 | 2.9 | 60 |

One μg $^3$H-labeled rye DNA (6700 cpm) was incubated 17 hr in 0.2 ml SSC at 60°C with rye or hexaploid wheat filter-bound DNA. Thermal stability profiles and $T_m$ values were determined after radioactivity eluted from guinea pig DNA-filters at each temperature was subtracted. Of the input $^3$H-DNA, 31% and 16% was eluted from the homologous rye DNA-filter(s) in the 27 μg and 7 μg filter-bound DNA experiments, respectively.

* Total cpm eluted from wheat DNA-filters are expressed as percent of total cpm eluted from rye DNA-filters (set at 100%).

TABLE 4

*Effect of formamide on DNA/DNA duplex formation*

| Percent formamide in solvent | $T_m$(°C) Tetraploid Wheat | Hexaploid Wheat |
|---|---|---|
| 30 | 75.2 | 75.1 |
| 50 | 78.6 | 78.6 |

One μg $^3$H-labeled tetraploid wheat DNA (3600 cpm) was incubated 16 hr at 43°C in 0.2 ml SSC containing 30% or 50% formamide with 15 μg tetraploid or hexaploid wheat filter-bound DNA. Thermal stability profiles and $T_m$ values were determined. Of the input $^3$H-DNA, 25% and 23% was eluted from the tetraploid and hexaploid wheat DNA-filters incubated in 30% formamide, respectively; these values were 13% and 15% with 50% formamide.

TABLE 5

*Relative percent binding of cereal grain DNAs to DNA-filters*

| Filter-bound DNA | $^3$H-labeled DNA Barley | Oats | Rye | Wheat |
|---|---|---|---|---|
| Barley | 100* | 19 | 58 | 72 |
| Oats | 12 | 100 | 15 | 17 |
| Rye | 59 | 22 | 100 | 100 |
| Wheat | 48 | 16 | 60 | 100 |

* Range was 92–105% and was typical.

One μg $^3$H-labeled DNA (4900, 2900, 11600, and 6600 cpm for barley, oats, rye, and hexaploid wheat, respectively) was incubated 17 hr in 0.2 ml SSC at 60°C with 7–8 μg filter-bound DNA. Radioactivity (0.1–0.2% of input) bound to guinea pig DNA-filters was subtracted. Of the input barley, oats, rye, and wheat $^3$H-DNA, 15.3%, 10.5%, 11.5%, and 9.2%, respectively was bound in the homologous case. The average of four determinations is given in the table.

great technical advantages in many cases. Table 4 shows that the $T_m$ of DNA/DNA duplexes formed in 50% formamide is about 3.5°C greater than that in 30% formamide. The conditions used in these experiments correspond to incubation in SSC at about 79°C and 64°C, respectively, according to the established relationship between reaction specificity and formamide concentration (McConaughy, Laird and McCarthy 1969). The failure to detect differences between tetraploid and hexaploid wheat DNA under these conditions will be discussed in the accompanying paper (Bendich and McCarthy 1970).

*DNA-filter duplex formation with cereal grain DNAs*: In order to compare the DNAs of the four cereal grains, duplexes were formed between labeled DNA and filter-bound DNA in all 16 combinations. This experiment establishes the relative quantities of duplexes formed under the conditions used, although it does not discriminate between duplexes of high and low stabilities. In Table 5 the homologous interaction is set at 100% and the relative heterologous binding is as indicated. The binding of labeled wheat DNA to rye DNA-filters (100%) is much greater than the binding of labeled rye DNA to wheat DNA-filters (60%). A similar result was obtained for the wheat-barley pair (72% and 48%).

This lack of agreement in reciprocal comparisons may be explained by the fact that plant DNA rates of duplex formation are greatly influenced by the size and number of families of related DNA sequences. Rye DNA-filters contained a sufficient concentration of sequences complementing the $^3$H-wheat DNA to allow a reaction equal to the homologous binding (9.2% of the input $^3$H-wheat DNA). Hence the fastest reacting portion of wheat DNA under these conditions of incubation is also present in rye DNA. But there are other fast-reacting sequences in rye which are not present in wheat in sufficient concentration, or absent. A similar explanation applies to the wheat-barley pair. Were the stringency of conditions of duplex formation to change, then the lack of reciprocal agreement in binding might increase or decrease depending on the distribution of the fast-reacting sequences. Similarly, increasing the fraction of $^3$H-DNA participation in duplex formation, by increasing either the DNA concentration or the time of incubation, could modify the agreement between reciprocal reactions. In previous work (Bendich and Bolton 1967) using the DNA-agar technique employing different criteria, there was a large discrepancy in reciprocal competition curves for the rye-wheat and rye-barley pairs, and a small one for the wheat-barley pair.

Thermal stability profiles of the 16 different duplexes are displayed in Figure 3. The mean thermal stabilities of duplexes between labeled barley DNA and the filter-bound DNA of barley, rye, wheat and oats decrease in that order. This implies that the linear DNA base sequence in barley more closely resembles that in rye DNA than in wheat or oats DNA, although the conclusion applies strictly to those sequences sufficiently similar to react. Analogous thermal stability curves using labeled oats, rye, and wheat are shown in Figure 3b, c, and d. On this criterion the DNA of oats is only distantly related to the other three DNAs. The duplex formed with oats DNA has the lowest stability in Figure 3a, c, and d. Table 6 summarizes the $\Delta T_m$ values derived from Figure 3, showing that they correlate well with the data of Table 5. Thus, labeled wheat DNA is decreasingly

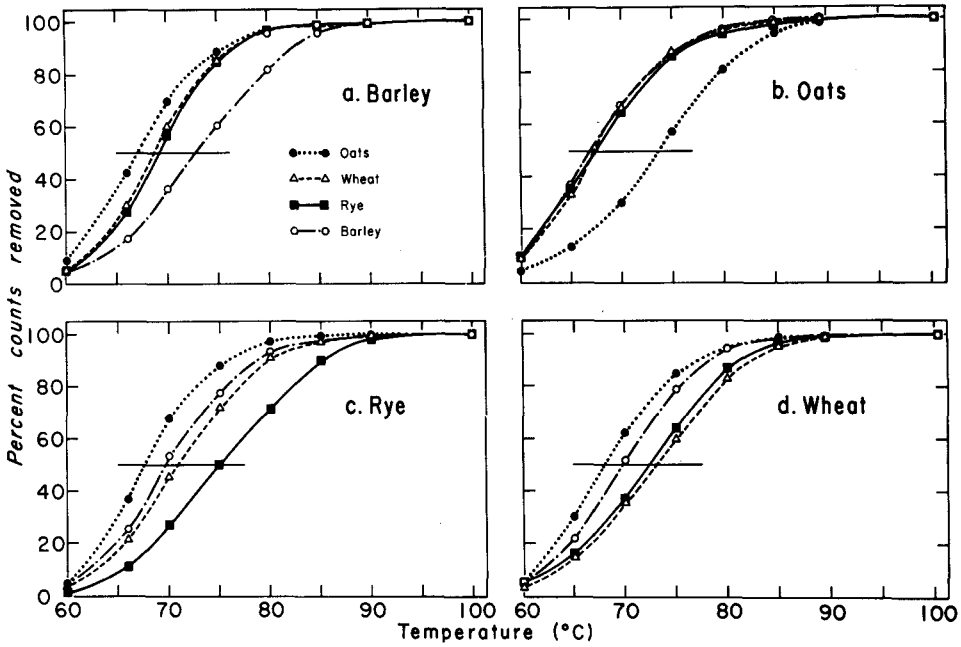


FIGURE 3.—Thermal stability profiles of DNA/DNA duplexes of some cereal grains. $^3$H-labeled DNA from (a) barley (b) oats (c) rye or (d) wheat was incubated with DNA-filters. Details are given in Table 6.

similar to the DNA of rye, barley, and oats. ($\Delta T_m$ values of 0.7, 3.4, and 5.0, respectively). The $\Delta T_m$ of barley-rye is lower than that of barley-wheat (3.6 *vs.* 4.0), and the $\Delta T_m$ of wheat-rye is lower than that of wheat-barley (0.7 vs. 3.4). Therefore, rye DNA is more closely related to both barley DNA and wheat DNA than barley DNA is related to wheat DNA.

TABLE 6

*Decrease in thermal stability of cereal grain DNA/DNA heteroduplexes*

| Filter-bound DNA | $^3$H-labeled DNA | | | |
|---|---|---|---|---|
| | Barley | Oats | Rye | Wheat |
| Barley | .. | 6.4 | 5.4 | 3.4 |
| Oats | 5.5 | .. | 7.2 | 5.0 |
| Rye | 3.6 | 6.1 | .. | 0.7 |
| Wheat | 4.0 | 6.1 | 4.1 | .. |

One $\mu$g $^3$H-labeled DNA (3200, 3200, 6700, and 4400 cpm for barley, oats, rye, and hexaploid wheat, respectively) was incubated 17 hr in 0.2 ml SSC at 60°C with 22–27 $\mu$g filter-bound DNA. A second incubation with 15–18 $\mu$g filter-bound DNA was used with $^3$H-oats DNA; these DNA-filters were combined with the first ones. Thermal stability profiles are shown in Figure 3. $T_m$ values (°C) were in the order barley, oats, rye, and wheat: 72.7, 67.2, 69.1, and 68.7 with $^3$H-barley DNA; 67.3, 73.7, 67.6, and 67.6 with $^3$H-oats DNA; 69.5, 67.7, 74.9, and 70.8 with $^3$H-rye DNA; 69.7, 68.1, 72.4, and 73.1 with $^3$H-wheat DNA. Radioactivity eluted from guinea pig DNA-filters at each temperature was subtracted. Of the input $^3$H-DNA, 40%, 26%, 31%, and 28% was eluted from the homologous barley, oats, rye, and wheat DNA-filters, respectively.

Figure 4 is an alternative presentation of the same data; it displays the relative amount of cross-reaction as well as the thermal stabilities. The area under each curve represents the quantity of duplex stable at and above the temperature of

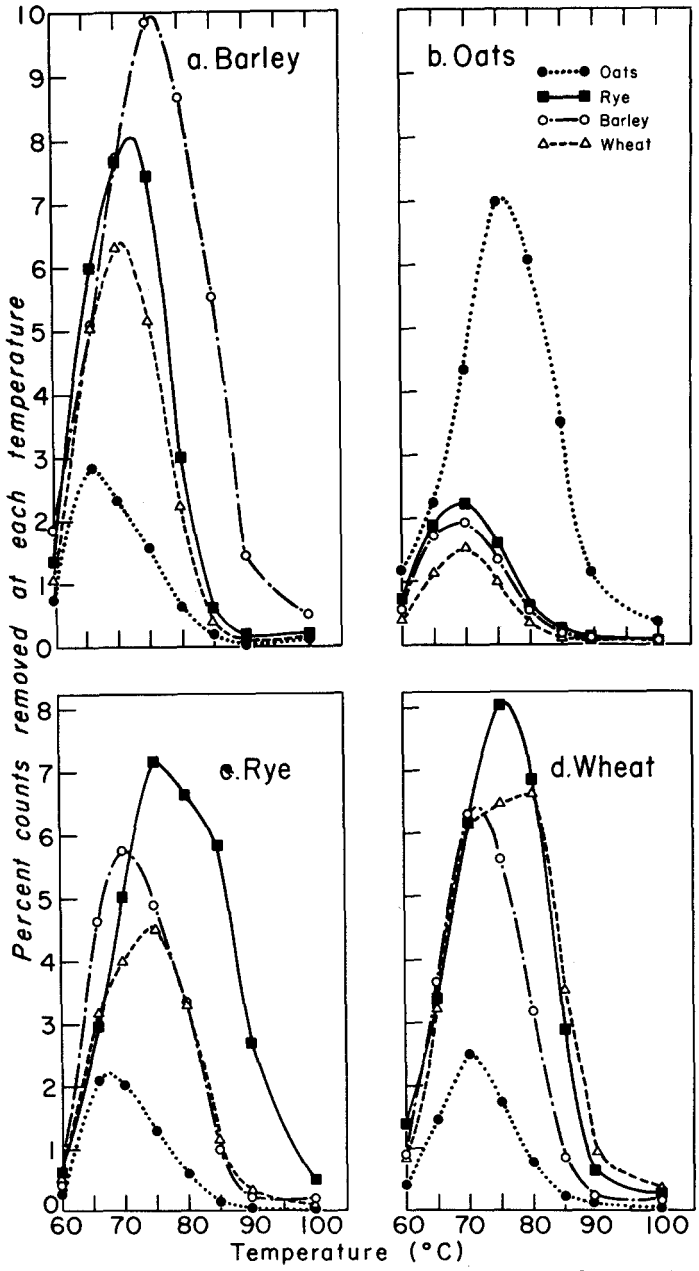


FIGURE 4.—Thermal elution of DNA/DNA duplexes of some cereal grains. Percent of input $^{3}H$-labeled DNA from (a) barley (b) oats (c) rye or (d) wheat eluted from DNA-filters at each temperature is plotted *vs.* temperature. This is an alternative presentation of the data in Figure 3.

incubation, and the shape of the curve is a function of the thermal stability of the duplex. For example, Figure 4a demonstrates that the barley-oats interaction is only about one-fifth that of barley-barley (calculated from the sum of counts removed at all temperatures) and reveals that the stability of the duplex formed is lower for interactions between barley and oats DNA. Again it is clear that oats DNA is only distantly related to the others.

Not all of the DNA in the incubation vessel participates in duplex formation. In the experiments summarized in Table 5 only 9–15% of the labeled DNA present was bound even to filters containing homologous DNA. In the thermal stability experiments this figure was 25–40%: this difference is due to the fact that 3–4 times as much filter-bound DNA was used in the thermal stability experiments. As will be demonstrated, similarity in the DNA of two species is maximal when the rapidly reacting DNA fractions are compared and minimal when longer incubation times are used. In the results presented to this point only the most rapidly reacting DNA fractions have been considered, and therefore the mean divergence in the total DNA is underestimated.

The diversity in DNA sequences among these four genera is much greater than that among biotypes containing the various genomes of hexaploid wheat (Table 4; BENDICH and MCCARTHY 1970). If we assume that the biotypes used in this study are representative of their genera, we may conclude from the above data that oats DNA is relatively distantly related to the others and that rye DNA is more closely related to the DNA of barley, oats and wheat than are any of the last three to each other. A schematic representation of the interrelationship in these grains based on the data presented here and in earlier work (BENDICH and BOLTON 1967) is given in Figure 5. Rye is seen to contain a genome which has diverged less from the DNA of the ancestral progenitor of these grains than has the DNA of the other three. This could mean that rye is either more primitive than barley, oats, and wheat or that it is representative of a stem line from which the other three have diverged.

*Reassociation of the DNA of cereal grains*: In experiments involving filter-bound DNA, the DNA concentration is not high enough to measure the slowly reacting portion of plant DNA. Much higher concentration of DNA in solution

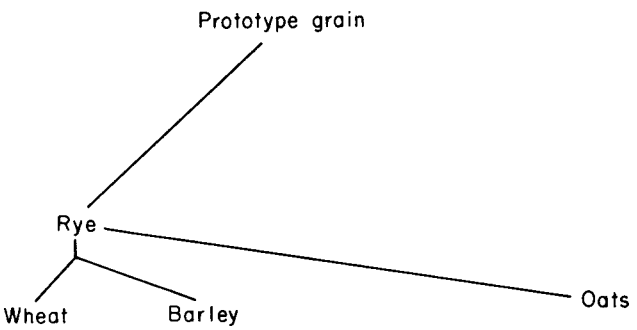


FIGURE 5.—Proposed phylogenetic relationships among the principal grains. The length of the lines between species is proportional to the divergence between the species.

and longer reaction times permit the DNA of complex organisms to reassociate completely (BRITTEN and KOHNE 1968).

Unlabeled wheat DNA containing a trace amount of labeled wheat or rye DNA was denatured and incubated in the presence of formamide. At various times, equivalent to the indicated $C_0t$ (initial DNA concentration × time) values, the reassociation of DNA strands was interrupted and the single and double stranded DNA fractions were separated on hydroxyapatite (HA). The kinetics of reassociation of wheat DNA at 40°C in 70% formamide with 0.78 M $Na^+$ and at 37°C in 48% formamide with 5 × SSC (which contains 0.975 M $Na^+$) are shown as $C_0t$ curves in Figure 6. These incubation conditions are equivalent to about 80°C and 61°C in SSC, respectively (MCCONAUGHY, LAIRD and MCCARTHY 1969). Wheat DNA reassociates much more rapidly in the lower

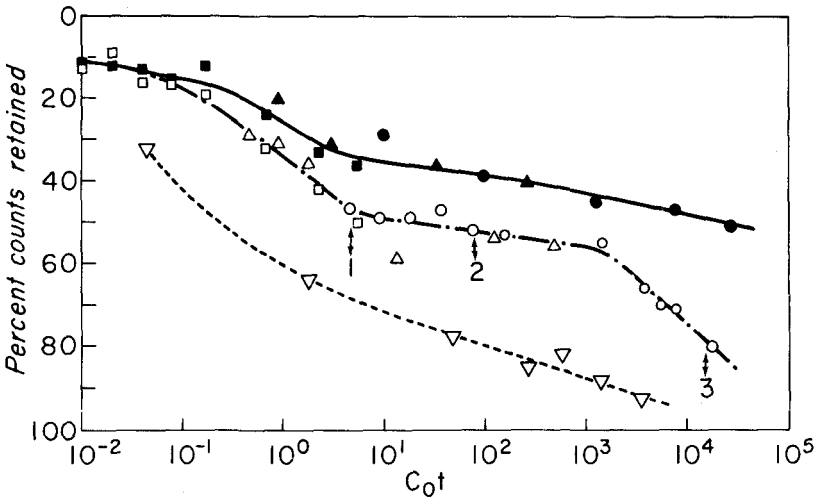


FIGURE 6.—Kinetics of DNA reassociation of wheat, and rye with wheat, measured with hydroxyapatite (HA). Samples of sheared DNA in 70% formamide and 0.78 M NaCl or in 48% formamide and 5 × SSC (inverted triangles) were sealed in capillary pipets and heated at 80–90°C for 5 min before quickly transferring them to a 40°C or 37°C (inverted triangles) water bath for incubation. At the indicated $C_0t$ values the extent of reassociation was measured with HA. Hexaploid wheat DNA at 4850, 485, and 11 μg/ml (circles, triangles, and squares, respectively) plus 800, 500, and 140 cpm of $^3$H-labeled wheat DNA (4400 cpm/μg; open symbols) or 6, 0.6, and 1 μg/ml $^3$H-labeled rye DNA (6700 μg/ml; filled symbols) was used. One hundred fifty to 250 cpm $^3$H-rye DNA was used per point. Wheat DNA at 1 mg/ml containing about 500 cpm $^3$H-wheat DNA per point is represented by the inverted triangles. The samples at 11 μg/ml contained $2.5 \times 10^{-3}$ M Na phosphate buffer pH 6.8; the pH of the 4850 and 485 μg/ml samples was near 7. At $C_0t$ values of 4.5, 78, and 14000 (arrows 1, 2, and 3) unlabeled wheat DNA samples were taken for optical melting curves (Figure 8).

■, ▲, ●: unlabeled wheat DNA + $^3$H-rye DNA incubated in 70% formamide and 0.78 M NaCl at 40°C.

□, △, ○: unlabeled wheat DNA + $^3$H-wheat DNA incubated in 70% formamide and 0.78 M NaCl at 40°C.

inverted triangles: unlabeled wheat DNA + $^3$H-wheat DNA incubated in 48% formamide and 5 × SSC at 37°C.

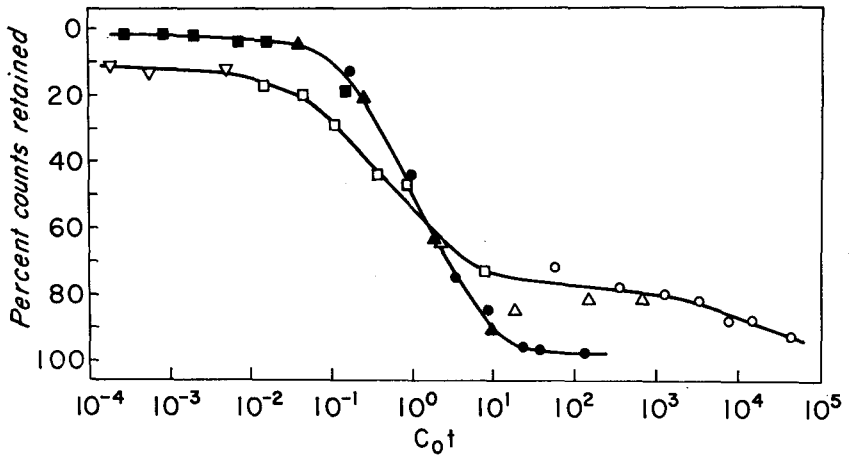


FIGURE 7.—Kinetics of DNA reassociation of wheat and *Bacillus subtilis* measured with hydroxyapatite. $^{3}$H-labeled hexaploid wheat (same as in Figure 6) and $^{14}$C-labeled *B. subtilis* (6700 cpm/$\mu$g) sheared DNAs were mixed and reassociated at 40°C in 62% formamide, 0.78 M NaCl and $5 \times 10^{-3}$ M Na phosphate buffer pH 6.8 as described in Fig. 6. Filled circles, triangles, and squares represent *B. subtilis* DNA at 16, 4, and 0.3 $\mu$g/ml, respectively. Open circles, triangles, and squares represent wheat DNA at 6000, 300, and 16 $\mu$g/ml, respectively. The inverted open triangles represent wheat DNA at 0.5 $\mu$g/ml reassociated in the absence of *B. subtilis* DNA. Two hundred to 500 cpm $^{14}$C and 300–500 cpm $^{3}$H were used per point.

formamide concentration (Figure 6). Under conditions of intermediate stringency, equivalent to 74°C in SSC, the rate of reassociation is between those corresponding to 80°C and 61°C (Figure 7).

These different reassociation rates may be explained as follows. The rate of DNA reassociation is maximal at 15–30°C below the $T_m$ (MARMUR and DOTY 1961; WETMUR and DAVIDSON 1968). This rate drops sharply at temperatures approaching the $T_m$ (i.e., 85°C in SSC). A second and probably dominant rate determinant is the fact that plants, like other higher organisms, contain DNA base sequences repeated many times. These multiple segments are not exact copies over long lengths of polynucleotide but belong to families of related sequences of varying degrees of similarity (BRITTEN and KOHNE 1968, Figure 8). Under stringent conditions, the number of segments sufficiently complementary in sequence to form a duplex is lower so that the reaction rate is decreased.

The $C_0t$ curves for wheat in the two higher formamide concentrations appear to be biphasic, whereas with the lowest formamide concentration only one broad curve is apparent. Theoretically, a $C_0t$ curve for a DNA lacking repeated sequences will have one component, the central two-thirds of which spans not much more than a factor of 30 in units of $C_0t$ (BRITTEN and KOHNE 1968). The $^{14}$C-*Bacillus subtilis* $C_0t$ curve in Figure 7 offers such an example. A curve broader than this limit would be representative of DNA containing families of repeated sequences. This kind of DNA will be termed "redundant" while nonrepeated sequences will be termed "unique." Under the low stringency condition of 61°C, the very broad $C_0t$ curve indicates that most or all of the base sequences in wheat

DNA appear to be redundant. But at the most stringent condition only 50–55% of the DNA is redundant. At the intermediate condition, the redundant component represents about 80%. For comparison, it should be noted that under conditions similar to the 61°C wheat $C_0t$ curve, DNA of rodents appears to contain 70–80% unique sequences (BRITTEN and KOHNE 1968; HENNIG and WALKER 1970), of artiodactyls about 60% (BRITTEN and KOHNE 1968), toad about 50% (DAVIDSON and HOUGH 1969), and Drosophila about 90% unique sequences (LAIRD and MCCARTHY 1968). We have also observed by optical measurements, that pea, barley, and rye DNAs reassociate much more rapidly than animal DNAs. Thus, the degree of internal homology within the genome of a higher plant is far greater than that apparent in animal DNA. It does not, however, necessarily follow from this generalization that more potential genetic information is present in animals than in plants, since these repeated DNA sequences are not exact replicas.

Wheat DNA was incubated to the $C_0t$ values of 4.5, 78, and 14000 as indicated by the arrows in Figure 6. The reassociated fraction in these three samples was

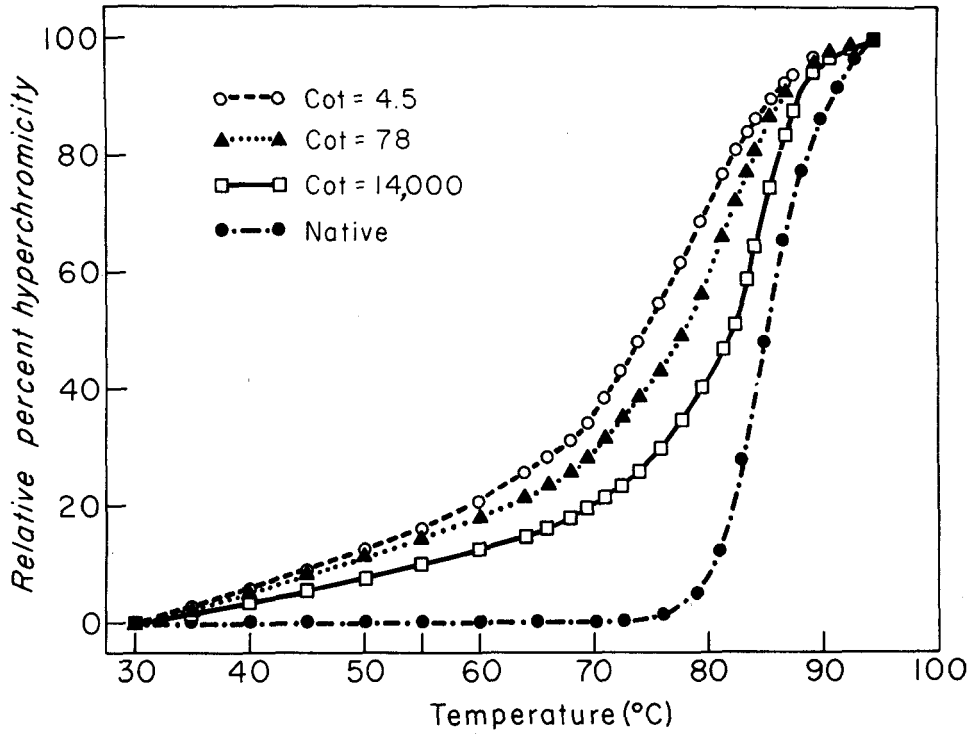


FIGURE 8.—Optical thermal stability curves of wheat DNA reassociated to various $C_0t$ values. Denatured hexaploid wheat DNA was incubated as described in Figure 6 to $C_0t$ values of 4.5, 78, and 14000 (see arrows in Figure 6). The reassociated fraction was recovered from HA and dialyzed to 1 × SSC before the measurements were made. The initial $OD_{260}$ ranged from 0.15–0.21; the hyperchromic effect was 22%, 27%, and 25% from the $C_0t$ = 4.5, 78, and 14000 samples, respectively. Native sheared wheat DNA (initial $OD_{260}$ = 1.2) had a $T_m$ of 85.2 and a 30% hyperchromic effect.

isolated and recovered from HA, and optical determinations made of the thermal stability. The thermal stability of the reassociated DNA complexes increased from low to high $C_0t$ (Figure 8) despite the fact that all three samples were "reassociated" according to the HA criterion. The melting curve for the wheat DNA incubated to a $C_0t$ of 14000 shows a component whose $T_m$ is close to that of native DNA. This strongly supports the proposition that the bulk of the reassociation of wheat DNA which occurs above $C_0t = 10^3$ in the 80°C equivalent curve in Figure 6 reflects true renaturation of unique DNA base sequences.

The rye-wheat $C_0t$ curve in Figure 6 parallels that of wheat-wheat up to a $C_0t$ value of about $10^3$. At this point the rye-wheat curve decreases only very slowly compared to the homologous curve. In comparying these curves it is obvious that rye DNA forms duplexes predominantly with the redundant fraction of wheat DNA. The continuing gradual drop in the rye-wheat curve may be due either to the reaction of labeled rye DNA with itself, or to a very low degree of homology between rye DNA and unique wheat DNA. This result implies that there is little, if any, base sequence homology between rye DNA and the unique base sequences in wheat DNA. In DNA-filter or DNA-agar experiments $C_0t$ values high enough to measure unique DNA sequences are not attained so that only the partially redundant DNA base sequences are measured. Since the unique DNA fraction does not cross-react, the DNA relatedness measurements using standard duplex formation techniques are overestimated. The minimal differences measured among the various genomes comprising that of hexaploid wheat using DNA-filters were amplified when incubations to high $C_0t$ values were used (BENDICH and MCCARTHY 1970). Earlier experiments also showed plants to be more closely related when redundant DNA fractions were compared (BENDICH and BOLTON 1967).

Figure 9a shows another rye-wheat $C_0t$ curve under conditions designed to duplicate the 80°C equivalent used in Figure 6. This curve has the same form as that in Figure 6, but the plateau region begins at higher $C_0t$. The most likely explanation of the differences in these curves is that 80°C is so close to the $T_m$ (85°C) that a slight change in formamide or salt concentration greatly alters the stringency requirements for strand reassociation. For example, if the formamide concentration in Figure 9a were actually 69% instead of 70%, then many rye DNA segments would reassociate with wheat which would be unable to do so in 70% formamide. This effect would be preferentially greater as the $T_m$ is approached. The rye $C_0t$ curve in Figure 9b has no plateau and there appears to be no unique DNA even at the 80°C equivalent condition. The shape of this curve is consistent with the presence of a broad spectrum of families of related sequences. The families would range from those with many members (reassociating at low $C_0t$) to those with few members (reassociating only at high $C_0t$).

The HA separation procedure can measure as little as 2% unreassociated DNA. This is evident for the *B. subtilis* curve in Figure 7. But even at the lowest $C_0t$ values for wheat and rye DNA (also found for barley DNA) 10% or more of the DNA binds to HA and is scored as reassociated. This value was reduced to 5% when a small volume of dilute DNA was denatured at very low salt con-

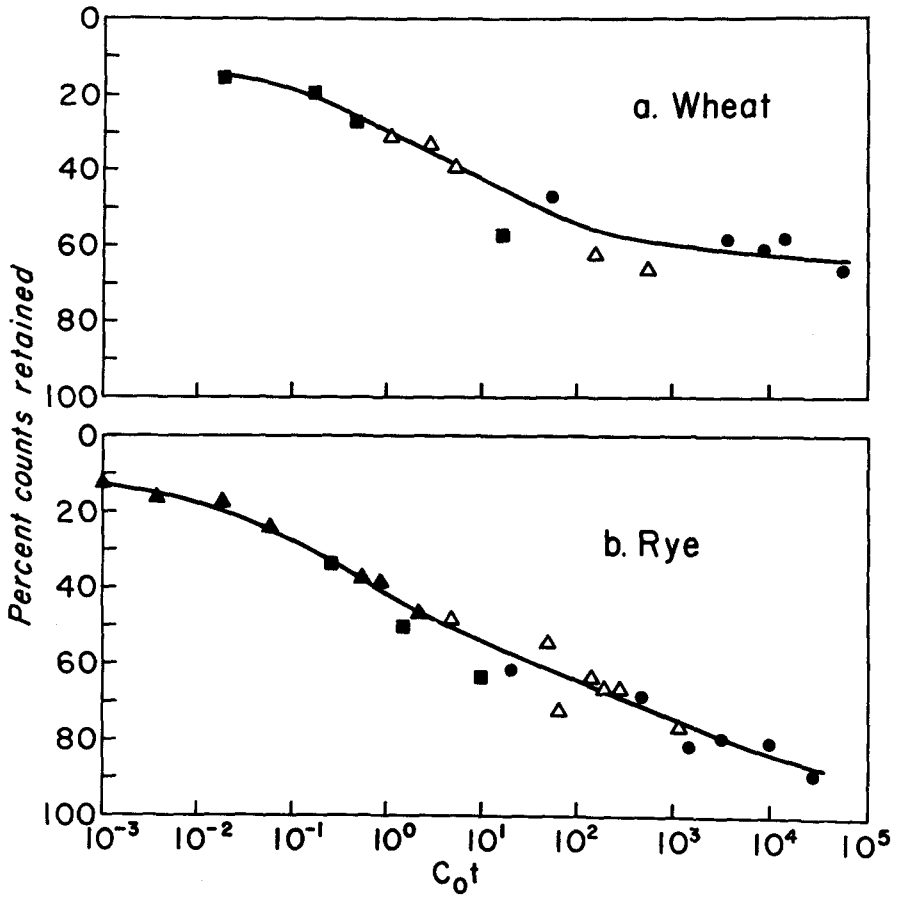


FIGURE 9.—Kinetics of DNA reassociation of rye, and rye with wheat, measured with hydroxyapatite. (a) $^3$H-labeled rye DNA (same as in Figure 6) was reassociated with excess unlabeled wheat DNA at 40°C in 70% formamide and 0.78 M NaCl under conditions designed to be identical to those in Figure 6. Filled circles, open triangles, and filled squares represent wheat DNA at 4850, 194, and 19.4 μg/ml, respectively and $^3$H-rye DNA at 9.2, 2, and 1 μg/ml, respectively. (b) Same as in (a) except unlabeled DNA was from rye. Filled circles, open triangles, filled squares, and filled triangles represent rye DNA at 2000, 87, 6, and 1 μg/ml, respectively. Two hundred to 800 cpm were used per point.

centration and quenched with excess cold buffer where strand reassociation should be precluded. Several explanations are possible. (1) These plant DNAs may be highly cross-linked so that complementary strands "snap back" on each other. (2) There may be some "hairpin" intrastrand reassociation. This would require base sequence inversions shorter than the length of the sheared strands. (3) Some reassociation may occur during HA fractionation. (4) There may be an extremely rapidly reassociating fraction similar to poly dAT in crab DNA (SUEOKA 1961) or mouse "satellite" DNA (WARING and BRITTEN 1966). Very

rapidly reacting DNA was also detected in studies of the reassociation of DNA from lettuce nuclei (WELLS and BIRNSTIEL 1969).

## CONCLUSIONS

The results obtained from comparisons of the redundant fractions of the various cereal grain DNAs are consistent with the general relationships outlined in Figure 1. The DNA of oats appears less similar to DNA of barley, rye, and wheat than are the last three to each other. Hence, these experiments support the assignment to separate tribes. The DNAs of wheat and rye are more related in base sequence than are either to barley, which is also consistent with the given sub-tribal segregation. The relationship between Aegilops and Tricium is discussed in the accompanying paper (BENDICH and MCCARTHY 1970).

These quantitative DNA comparisons lead to the scheme in Figure 5 which proposes that rye may be closer to the stem line for evolution of these grains and, therefore, a more primitive member of the family. The data in Table 6 show the $\Delta T_m$ for the $^3$H-rye/wheat duplex to be 4.1°C, while that for the reciprocal duplex, $^3$H-wheat/rye, is 0.7°C. This type of result can be attributed to different extents of divergence of DNA base sequence among individual members of sequence families in the two species. For example, the failure of these reciprocal $\Delta T_m$ values to agree may be explained as follows. Of the DNA sufficiently similar between rye and wheat to form a duplex under the conditions used, the members of the families of related polynucleotide sequences have diverged from one another to a lesser extent in rye than in wheat DNA. The duplexes between $^3$H-wheat DNA and rye DNA-filters are of a stability approaching those of wheat/wheat duplexes since the spectrum of dissimilarity among the divergent wheat sequences is wide. The duplexes formed between $^3$H-rye DNA and wheat DNA-filters are much less stable than corresponding rye/rye duplexes again since the reacting sequences in wheat are more divergent than in rye DNA. The fact that the absolute $T_m$ of the rye/rye duplex is greater than that for wheat/wheat (Table 6, legend) supports this analysis. An analogous explanation applies to the rye-barley pair (in Table 6 the $^3$H-rye/barley $\Delta T_m$ is 5.4°C and the $^3$H-barley/rye $\Delta T_m$ is 3.6°C).

Assuming that rye is a more primitive member of the Gramineae, these results are consistent with the following course of events occurring during the speciation of this group of cereal grains. After the generation of large families of redundant base sequences in the primitive species, the more specialized members of the Gramineae were formed by elimination of part of the DNA (STEBBINS 1966) and concomitant divergence among the similar base sequences comprising the families. This would explain the nonreciprocal results obtained in the various DNA comparisons. Moreover, this increased intragenome base sequence divergence would lead to an increase in the fraction of DNA of unique base sequence evident in the more specialized species. It may be relevant to note HINEGARDNER's (1968) report that specialization among teleost fishes has been accompanied by a loss of DNA.

From the $C_0t$ at 50% reassociation the molecular weight of a DNA may be calculated relative to a known standard (BRITTEN and KOHNE 1968). This

computation is not possible for the unique wheat DNA in Figure 6 without a standard for comparison. Since the $^{14}$C-*B. subtilis* and $^{3}$H-wheat DNAs were mixed for the curves in Figure 7, we can use $C_0t = 1$ for 50% reassociation of this DNA whose molecular weight is about $1.3 \times 10^9$ daltons (YOSHIKAWA 1968). The unique wheat DNA has a 50% reassociation of about $C_0t = 15000$, and represents about 20% of the total of these conditions. Its molecular weight would, therefore, be $15000 \times 1 \times 0.2 \times 1.3 \times 10^9 = 3.9 \times 10^{12}$. That of all the haploid (21 of the 42 chromosomes) DNA would be $3.9 \times 10^{12} \times 5 = 19.5 \times 10^{12}$. This estimate of about $20 \times 10^{12}$ is a crude one due mainly to an uncertainty in estimating the 50% reassociation $C_0t$ for the unique fraction of wheat DNA in this case, but is considerably greater than the size of mammalian genomes (MCCARTHY 1965).

There are two main conclusions to be drawn from the hydroxyapatite experiments. (1) Plant DNA is characterized by a high proportion of repeated sequences, more so than animal DNA. (2) There is no one number ascribable to the relatedness of two plant DNAs. This was also evident from the DNA-filter data. However, we may say that the most redundant DNA fraction appears to be more closely related in two plants than is the less redundant and unique DNA. This same conclusion seems also to be valid for mammalian DNA (BRITTEN and KOHNE 1967; LAIRD, MCCONAUGHY and MCCARTHY 1969).

## SUMMARY

Interspecific associations of the DNA of barley, oats, rye, and hexaploid wheat *in vitro* were employed as criteria for relatedness and evolutionary history. The thermal stability and extent of formation of DNA/DNA duplexes were measured for each of the 16 pairwise combinations. These comparisons, which measure only the DNA of partially repetitious base sequences (redundant DNA), showed that rye and wheat share more DNA base sequences than does either with barley. Similarly, oats DNA is only distantly related to the others. Rye DNA appears to be more closely related to the DNA of barley, oats and wheat than are any of barley, oats, and wheat related to each other. The data suggest that rye is a more primitive member of the grass family than barley, oats or wheat. The DNA of higher plants is characterized by a high proportion of redundant DNA, more so than animal DNA. The proportion of wheat DNA which appears in redundant sequences is not an intrinsic value, but depends upon the conditions of measurement. The similarity of wheat and rye DNA was greater when redundant DNA was compared than when nonrepeated DNA sequences were compared. A larger fraction of the rye genome is composed of redundant DNA than is the case in wheat. We propose that specialization in higher plants involves an elimination of DNA as well as sequence divergence of repeated DNA base sequences.

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