Methods of obtaining duplications

Chromosome segments may become duplicated following insertion in another location either in the same chromosome or in a non-homologue. Also the interchange of terminal segments of different lengths from the same arm of a pair of homologous chromosomes is expected to produce a duplication, as described by Beard (1960). These exchanges may be produced by irradiation of diploid cells only, whereas the insertion of a segment in a new location may be produced by irradiation of diploid or monoploid cells.

Duplications for known loci may be produced by selection among the progeny of crosses between interchanges involving the same chromosomes, as described earlier (p. 107) (Gopinath and Burnham). Another source is among the progeny of crosses between overlapping or included inversions as described on page 47 (cf. Sturtevant and Beadle 1936). Either of these methods requires detailed knowledge of the positions of loci to be duplicated and requires interchanges or inversions with breaks at the proper points.

Methods of obtaining doubled chromosome numbers

Natural occurrence

Occasional polyploid cells or even sectors may be found in the tissue of untreated diploids. In cases where polyploid pollen grains may be recognized under the microscope by their large size, or by a different number of lobes, or germ pores, it has been found possible to use them for pollination. They may be picked up and transferred by means of a horsehair or glass tubing drawn out to a very fine thread. Pratassenja (1939) in peaches obtained 7 triploids and 15 diploids from 30 seeds produced by 1202 such pollinations. It is thought that such polyploid cells are the results of normal division or reproduction of the chromosomes followed by failure of a cell division and the formation of a restitution nucleus. The frequency of diploid spores (or gametes in animals) might be increased by temperature shocks or other treatments as will be described later.

Since tetraploids are often giant in size or show higher sterility, a search for them in large populations has a chance of success.

Genes causing complete asynapsis at meiosis resulting in polyploid gametes and offspring have been reported by Beadle (1930, 1933) in corn, by Bergner, Cartledge and Blakeslee (1934) in Datura, by Beasley (1942) in cotton, by Li et al. (1945) in wheat, by Sansome and Sansome (1940) in Pisum, and by Gowen (1933) in Drosophila (gene C3G). The gene for elongated chromosomes in corn (Rhoades 1956) also produces 2n spores. These genes may be introduced into any desired variety, but would have to be removed later from the polyploids produced. If these stocks are crossed with tetraploids, the full-sized seeds have a good chance of being tetraploid (cf. Alexander, 1957).

Although twin seedlings are usually both diploid, a small percent may include a triploid, and occasionally a tetraploid or a haploid (Müntzing 1938, Kasparyan 1938 and others). In winter and spring wheat the frequency of twins is about 1 in 1,000 plants. Seed-labs in which workers have been alerted to select twin seedlings have been utilized in some cases. In corn there is a recessive gene siamensis (sn) in chromosome 6 which produces twinning, but these have only the diploid number.

Cell regeneration

Many attempts have been made to induce polyploidy. Élie and Émile Marchal (1907 and later) induced polyploidy in mosses by cutting the diploid sporophyte and keeping it under moist conditions. The cells at the cut ends regenerated threads which were a true

protonema and produced diploid germ cells. In certain cases such germ cells were not functional but in others they functioned to produce a 4n sporophyte. Von Wettstein (1924) has reported the production of 8n gametophytes by this method. Tetraploid sporophytes have been obtained in the same manner in certain ferns, e.g. Lawton (1936) in Osmunda and Cystopteris and Partanen (1961) in Osmunda.

Winkler (1916) grafted tomato on nightshade and 10 days later cut them off at the graft level primarily for the production of various types of chimeras. Among the shoots developed from the callus tissue some were found to be tetraploid. It is now known that callus tissue which forms on the cut ends of certain plants, especially Solanaceae, contains polyploid cells; and that certain of the adventitious buds which are formed from that tissue are polyploid. Jorgenson (1928), Sansome (1930) and others have used this method for species of Solanum. They found about 6% of the adventitious buds had double the chromosome number. Lindstrom and Koos (1931) found binucleate cells in the callus tissue in the tomato. They used the callus method to induce diploidy in a haploid tomato, and in turn to induce tetraploidy in the diploid thus obtained. They obtained 32 and 36% respectively of polyploid shoots. Thus they were able to produce strictly comparable homozygotes at the haploid, diploid and tetraploid levels. Greenleaf (1938) reported the successful use of lanolin paste containing 1% of indole-3-acetic acid (hetero-auxin) in inducing abundant callus formation on decapitated stems of certain Nicotiana species and amphidiploids. Of 1,173 plants established, 13% were tetraploid and about 1% were octoploid. In certain other species of Nicotiana and also in tomato, this treatment was not effective. In certain cases, scraping the upper 2 to 3 leaf axils before treatment was more effective than decapitation in producing adventitious shoots. Povolochko (1937) found that the pollinia of a tropical orchid stimulated regeneration in Nicotiana.

In a sterile form of Pelargonium roseum, removal of all axillary buds from cuttings gave regeneration of shoots from the callus at the base of the cuttings. Those that had the doubled chromosome number were fertile (Shchavinskaya 1937).

In cabbage, Shchavinskaya (1937a) was able to induce polyploidy by decapitation and removal of the axillary buds. Regeneration at the cut end was favored by high moisture obtained e.g. by means of a ball of peat moss on the cut end. Low temperature may also have favored it. No callus was formed and the period for regeneration was 27 to 30 days. Only 25% of the decapitated plants regenerated and only 1 to 2% of these had the doubled chromosome number.

Kostoff and Kendall (1929) found irregular meiosis in flower buds of Lycium halmifolium Mill. attacked by gall mites. Kostoff and Kendall (1933), Winge (1927), and many others have reported multinuclear or polyploid cells in galls produced by various agents, e.g. bacteria, nematodes, or tumors caused by chemical substances. Levine (1931) reported chromosome numbers from haploid to tetraploid and higher in carcinomas of fowl, rat and man.

Wipf and Cooper (1938) reported that in the nodules of red clover, common vetch and garden pea the infected cells are regularly tetraploid. They advanced the hypothesis that only tetraploid cells could become infected, but it may be that the bacteria induced the change.

Physical Agents

TEMPERATURE SHOCKS. Extreme temperature changes were found to result in a higher frequency of polyploid cells. Belling and Blakeslee (1924) found tetraploid branches in Datura as a result of cold treatment.

Randolph (1932) successfully produced tetraploids in corn by treatment with temperatures of 38°-45°C at the time of the first division of the zygote. The frequency of tetraploids ranged from 2 to 5%. He used an electric heat pad to apply the heat locally to the ear; or else treated the entire plant in a heated room (described and used also by Dorsey, see below). Brink (1936) described a portable chamber for applying the heat treatment. Peto (1938) has described a special method also. High temperatures have produced chromosome doubling in barley, rye and rice (Matusima 1935). Dorsey (1936) reported 15 instances of chromosome doubling among 426 plants obtained from heat treated seeds. Doubling was obtained in Triticum durum Desf., T. polonicum L., T. vulgare Vill., Secale cereale L.; and the two hybrids, T. vulgare x T. compactum Host. and T. vulgare x S. cereale. The plants to be treated were subjected to 25°C for 20 hours, then to 43°C for 20 to 30 minutes, after which they were returned to the greenhouse. Atwood (1936) obtained one tetraploid and one triploid sweet clover among 575 seeds. Peto induced tetraploidy in barley (1936) and in wheat (1938) by means of heat treatment.

Lutkov (1937) reported in Raphanobrassica (the doubled hybrid of radish and cabbage) that a temperature of 0° to 1.5°C for 24 hours produced diploid and tetraploid pollen, 6.62% and 2.34% respectively. A treatment of 1 1/2 to 1 3/4 hours resulted in 37.6 to 55.8% of diads at the quartet stage. Sax (1936) reported in Rhoeo that temperatures of 10°C for 2 to 3 days, then at 36°C for 1 day gave the greatest number of polyploid microspores.

Lutkov (1937) reported chromosome doubling by use of low temperature and chloroform on flax.

CENTRIFUGATION. Changes in chromosome number produced by centrifuging seedlings of Nicotiana were reported by Kostoff (1935). The extra chromosomes were fragments in some cases.

In our laboratory we were unable to find any abnormalities which survived to the sporocyte stages in barley or maize, although the effects in the seedling stage ranged from none to complete killing (Phinney, unpublished). An ordinary centrifuge, one with temperature control, and an ultracentrifuge were used. Centrifuging of silkworm (Bombyx mori) eggs at the time of fertilization and at the first division of the zygote was reported by Kawaguchi (1936) to produce triploids, tetraploids, and hexaploids. In one experiment, of 626 males, 12% were approximately 3n and 1.7% were about 4n.

X-RAYS. X-rays applied to seeds and seedlings of rye were reported by Breslavetz (1939) to produce polyploids.

Colchicine

Blakeslee (1937), followed shortly by Nebel (1937) working independently, reported the successful use of colchicine in the production of polyploidy. According to Blakeslee's account, in June of 1937 he had initiated experiments to induce chromosome doubling by chemical treatment; and had included colchicine at the suggestion of O. J. Eigsti. For an account of the chain of events leading up to these publications, see Eigsti and Dustin (1955). The first studies of the effects of colchicine on mitosis were made on mice at Dustin's laboratory in Brussels.

The effect of colchicine on mitosis was discovered when it was included with other alkaloids in experiments on cancer chemotherapy (Cf. Eigsti and Dustin, 1955).

The results obtained were spectacular. Blakeslee (1939) reported the successful production of polyploids in 48 different species, including 29 genera and 16 families of

seed plants. From 1938 to 1942, autopolyploid strains of all the major agricultural species of Sweden were produced (Levan (1949), Turesson (1946), and Åkerman et al. (1948)). Very few of these appear to have commercial possibilities. It was used also in many laboratories to double the chromosome number of sterile hybrids, producing amphiploids. Some of these had been known before, but colchicine made it possible to accomplish their production on a large scale. For example, according to Eigsti and Dustin (1955) more than 80 different amphiploids involving the 14-, 28-, and 42-chromosome wheats were produced in Russia (cf. Zhebrak 1944). Bell and his group at Cambridge, England have produced a large number in wheat and related genera also (Bell 1950, Bell and Sachs 1953). Also Kihara and his group in Japan (Kihara et al. 1950) and Sears in the United States (Sears 1939a, 1941) have produced amphidiploids in wheat by treatment with colchicine.

Colchicine was effective also on Marchantia, a liverwort (Blakeslee 1939). Autopolyploidy induced in Penicillium notatum by colchicine has been reported (Gordon and McKechnie 1946). Certain fungi and bacteria utilize it as a source of nitrogen. A Pythium causes a disease of Colchicum in spite of the fact that Colchicum is one source of colchicine (Moore 1940).

Colchicine has induced other changes which include gene mutations (see page 261), and interchanges.

SOURCES AND CHEMISTRY OF COLCHICINE, AND MEDICAL USES (cf. Eigsti and Dustin 1955). Colchicine, a poisonous chemical, is extracted from the seeds and bulbs of the wild meadow saffron or autumn-flowering crocus, Colchicum autumnale L., which is widely distributed in Europe and in Great Britain. It is sold as a flowering bulb in the U. S. The seeds contain from 0.2 to 0.8% of colchicine, the corms from 0.1 to 0.5% and there is a slight amount in the flowers. It also occurs in the tubers of other members of the Liliaceae.

Colchicine may be identified microchemically with platinirhodanide (British Chem. and Physiology Abstr. 1931:778). It is also fluorescent, a fact used in studies of its movement in treated plants. Pure colchicine is $C_{22}H_{25}O_6N$. It has a tricyclic chemical structure with two fused 7- membered rings. Colchicine is readily soluble in alcohol, chloroform, or in cold water, but is less soluble in hot water. Other allied compounds are associated with it in the plant. One of these, compound "F" is less toxic than colchicine yet more active in blocking mitosis.

The Byzantines used colchicum as a remedy for relieving pain connected with gout; occasionally with fatal results. It has been listed as a gout remedy in the U. S. Pharmocopea. There are extensive side effects which thus far make it dangerous to use.

Tumors on plants have been killed by applications of colchicine (Brown 1939, Dermen and Brown 1940). Results on tumors in animals have not been consistent.

ACTION OF COLCHICINE ON MITOSIS. Nebel (1937), Dermen (1938) and Levan (1938, 1939) reported that colchicine produced its effects by inhibiting spindle formation and preventing anaphase. The pairs of longitudinally split metaphase chromosomes have the appearance of pairs of parallel rods which are shortened and swollen. This appearance has been termed a "colchicine- or C-mitosis" by Levan. Meyer (1943) and others have used colchicine as a pre-treatment to aid the counting of chromosomes. Under the influence of the drug, the divided chromosomes in a cell remain in a single restitution nucleus. If colchicine is still present, the chromosome number may double and redouble

at the divisions preceding the archesporial cell, thus forming tetraploid or octoploid pollen mother cells. Pollen mother cells at the prophase stages showed asynapsis and abnormal bivalents. If the first meiotic division were prevented, tetraploid pollen monads were formed. If only the second division were prevented, diploid pollen diads were formed. If the first division of the microspore were affected, diploid nuclei in the pollen grain were formed. In root tips of Allium cepa L., up to 500 to 1,000 C-pairs were estimated in one cell, the normal number being 16 (Levan 1939).

Dermen (1938), in comparing its effects with those of temperature shocks, reported that temperature changes produced clumping and fragmentation of the chromosomes, while colchicine did not. He suggested that colchicine acts on the division forces of the cytoplasm while the temperature effect is a specific one on the chromosomes.

GENERAL CONSIDERATIONS. Colchicine appears to affect only the dividing cells. Hence the most effective treatments are those applied when the cells of the tissue are most actively dividing. In the growing points, even at the most optimum time all the cells do not divide simultaneously. Successive treatments every few hours may be more effective in polyploidizing a large proportion of the cells than treatments given at longer intervals. The treatment results in sectorial chimeras of diploid and polyploid tissue. If the polyploid sector is too small the diploid tissue may outgrow it. This competition varies in different species and may very well vary with the genotype within a species. In perennials propagated asexually the problem is to establish polyploids from the mixoploids produced. Hill and Myers (1944) reported that 2n and 4n lines of ryegrass (Lolium perenne L.) could be established from such plants by vegetative propagation, although some persisted as mixoploids through 11 such vegetative generations. Brewbaker (1952) has reported similar experiences with white clover. Where propagation is by seeds, the pollen from such mixoploids will contain some 2n grains. If these are enough larger to be distinguished under low powers of the microscope, pollinations may be made with individual grains picked up with a horsehair or other means.

At the Aberystwyth station, the practice with red clover has been to examine the pollen of individual heads, then to cross those heads which had a high proportion of 2n pollen.

Bates (1939), following up a suggestion made by Kostoff, examined several sites of Colchicum colonies for the presence of naturally occurring polyploids. Although one colony was at least 20 years old, no abnormal-appearing species were found. He suggested that competition would have eliminated any such plants. It is also possible that fungi and bacteria may have broken down the chemical. A test of a soil sample might be made for the presence of colchicine or for its effect on seedlings.

Kostoff (1939a) reported that seedlings grown on or in the pulp of Colchicum showed swellings similar to those induced by low concentrations of colchicine.

Cornman (1942) has reported that stronger doses are necessary to induce polyploidy in Colchicum itself.

EFFECT OF COLCHICINE AND TEMPERATURE SHOCKS IN ANIMALS. Pincus and Waddington (1939) reported the production of tetraploid ova in rabbits by the use of 0.00041% colchicine on newly fertilized eggs. These ova failed to cleave or did so at a lower rate. Artificial insemination with sperm to which colchicine was added has

been used in rabbits and pigs. Melander (1950) reported in rabbits that one offspring had the 3n number in certain mitotic cells of the testes, 2n in others. Häggquist (1951) and Häggquist and Bane (1950) have reported results on induced polyploidy in rabbits and swine. Colchicine treatment was applied at the moment of fertilization. One male pig had 47 chromosomes consistently in mitotic figures (32 + 15 chromosomes). Of 7 young in rabbits, one ♀ and two ♂ lived nearly a year, four died at 8 weeks or before. Erythrocytes and the sperms of the ♂ were larger. In mice 3n and 4n zygotes were reported after abnormal temperature treatment at an early stage. Higher, but different numbers of chromosomes were observed in cells of bone marrow and testicles.

In stickleback fishes (about 55 mm. long), Gasterosteus aculeatus L., triploids have appeared among the progeny following high or low temperature shocks (Swarup 1959). The most effective method was the treatment of eggs at 0° C for 1 1/2 to 3 hours, starting three minutes after insemination. Triploidy was induced in about 50% of the eggs. The triploids were vigorous and required less oxygen than the diploids (Swarup 1959a).

Methods of using colchicine in plants

Different species vary greatly in their susceptibility to the action of colchicine. Directions for a few of the methods that have been used successfully in the production of polyploids will be given here. For more details, see Dermen (1940), Krythe and Wellensiek (1942), Bremer-Reinders and Bremer (1952), Eigsti and Dustin (1955), Elliott (1958), and Dermen and Emsweller (1961).

Aqueous solutions or emulsions may be used, or colchicine may be mixed with agar or lanolin. Wetting agents have added to their effectiveness. Glycerine may be added to the solution also.

SEED TREATMENT. For rapidly germinating seeds, soak in a solution for 1 to 10 days before planting. The reported range of dilution is from 0.001 to 1.6%, usually 0.2% is effective. Soak in a shallow container for aeration. For seeds which germinate slowly, defer the treatment until the seeds commence active germination. Myers (1939) reported success in perennial rye-grass by placing the dry seeds on blotting paper moistened with 5.5 cc of colchicine solution, then transferring them to water-moistened paper.

SEEDLING TREATMENT. Immerse freshly germinated seeds in colchicine solution in a shallow container for 3 to 24 hours. Methods which avoid immersion of the roots have been devised. Thompson and Kosar (1939) germinated lettuce seed in the dark on filter paper, then inverted it so only the stem ends of the seedlings were immersed in colchicine solution. The treatment was repeated after 16 to 20 hours. Dermen and Emsweller (1961) suggested that the root ends of seedlings be placed on a strip of absorbent cotton that is thoroughly wet with water and then the seedlings rolled into a bundle. The bundle is inverted and only the stem ends immersed in the colchicine solution in a vial.

TREATMENT OF GROWING SHOOTS AND BUDS OF HERBACEOUS PLANTS. Brush or drop the colchicine solution on the exposed tips, or immerse them for several hours in the solution. A 0.5 to 1.0% mixture of colchicine in lanolin may be smeared on them. This latter treatment may need to be applied 2 or 3 times per week. Frandsen (1939) produced tetraploids by using 1% colchicine in lanolin paste smeared on the upper part of the mature beet root. Rasmusson and Levan (1939) found that later treatments of the flowering stalk in sugar beet were more successful than earlier ones. The 4n tissue then had less time to be overrun by 2n tissue before flower formation. Abegg (1941) produced tetraploid sugar beets by applications of drops of colchicine many times daily to the growing points of seedlings.

Solutions of colchicine mixed with agar may be poured into gelatin capsules which have been waterproofed with collodion, and the capsules then inverted over the growing points. Another method has been to thread a colchicine saturated cotton twine into the plant with a needle.

SPECIAL METHODS FOR CEREALS, GRASSES. It has been more difficult to induce polyploidy which would continue to maturity in the grasses and in certain other monocots. Special methods have been successful. Sears (1941) has reported the production of polyploids in wheat by placing absorbent cotton saturated with an aqueous colchicine solution or a lanolin plus colchicine paste on the crown of well-tillered plants. He was most successful in the trials using the aqueous solution, and also obtained no tetraploid sectors unless the plants were kept in a moist chamber for 2 to 5 days.

A comparison of several methods on wheat was reported by Bell (1950). In one of the most successful methods the tillers of young plants were cut off an inch or so above the growing points. Then tightly-fitting capillaries filled with colchicine solution were slipped over them, and held up by a wire pushed into the ground, the upper end of the wire being wound around the capillary. Plastic straws are a good substitute, vaseline being used to seal the lower end around the stem. This method was successfully used in our own lab on a Triticum timopheevi x Khapli emmer hybrid. There was a fertile sector with 3 seeds on one head. The method was not successful on barley (Tabata, unpublished). Some of the treated plants had very broad leaves, but this seems to have been a hormonal effect, as the progeny were normal (Levan 1942).

There seems to be a greater chance of success when treating sterile hybrids. However, polyploidy in that material is much more easily recognized since it commonly restores fertility.

A method used by Gerrish (1956) for maize was to introduce the colchicine under partial vacuum. Germinating kernels were suspended from a string in an erlenmeyer flask so only their coleoptiles (open at tip end) were submerged in the colchicine solution. The cork with the hose attachment to the vacuum pump was inserted and the air exhausted. When the air had been removed, release of the pressure filled the coleoptile with colchicine. The seedlings were then transferred to nutrient solution for a few days before planting.

For rice, Luong (1951) split the upper portion of the seedling lengthwise to the growing point, then inserted a small wad of colchicine-soaked absorbent cotton or preferably .5 mm squares of blotting paper into the split. Drops of colchicine solution were applied at that point.

METHODS FOR WOODY OR SEMI-WOODY PLANTS. A somewhat stronger solution appears to be necessary for woody plants. Use a 1% colchicine solution in a 10% aqueous solution of glycerine, or a 1% aqueous solution of colchicine. A very small amount of a wetting agent may be advisable to give better wetting and penetration, the amount to be determined by trial. Treat only 1 or 2 buds at a time, removing the other buds at least two days before treatment to force the remaining ones into more active growth. The bud scales and young leaves should be cut away to expose the growing points. The glycerine solution may need to be applied only at 1- or 2-day intervals, but the aqueous solution should be applied twice a day for 3 consecutive days on rapidly growing material, and over a longer period of time on slowly growing material.

As described by Dermen and Emsweller (1961), leaves that are denser in texture, darker in color, larger or broader than normal, or partially changed in this manner "should be looked for in the upper portion of shoots that develop from the main growing

point of the treated tips or from axillary buds that were present in the treated regions. Polyploid branches may be grown from buds in the axils of the changed leaves by cutting off the part above these leaves." "Any extraneous growths that may hinder growth in the treated part should be constantly eliminated." When a polyploid sector is discovered in an otherwise normal stem, attempts should be made immediately to force it to grow out into a branch; otherwise the polyploid sector may be lost as the stem develops.

Tetraploidy has been induced in Carica papaya (Hofmeyr and van Elden (1942), Sequoia gigantea (Jensen and Levan 1941), apples (Dermen 1952), grapes (Dermen 1954), and other woody plants.

McKay et al. (1945) have reported applying colchicine by the aerosol method, offering the suggestion that by this method other agents could be added to increase penetration into tree buds and other meristems difficult to affect by ordinary methods.

METHODS FOR BULBS, CORMS, RUNNERS. The colchicine may be introduced into the growing regions by a hypodermic needle. For gladiolus bulblets, a partial vacuum has been used to secure penetration (Manley 1941). In lilies where small bulbs form at the base of the scales which are detached from the bulb and planted, immediate soaking of the detached scales in a 0.2% solution for about 2 hours followed by planting with the tips exposed has induced polyploidy (Emsweller and Brierley 1940).

Other chemical agents

While colchicine has been the most efficient chemical in the production of polyploid plants, many others appear to produce similar effects on cells. One that has been effective on certain species is acenaphthene, an inexpensive chemical. Acenapthene was reported by Kostoff (1938b), Nawaschin (1938), Shmuck and Gusseva (1939) to cause thickened roots and leaves of grass seedlings. Because it is relatively insoluble, seedlings were grown on filter paper in petri dishes and sprinkled with acenapthene crystals and the treatment continued for 4 to 5 days. Fatalizade (1939) produced Nicotiana polyploids by using this compound. Success was reported in wheat by having the stems grow up through a lamp chimney or glass cylinder prepared by subliming on the inside a layer of acenapthene crystals. The exposure during development apparently was sufficient to induce polyploidy.

Mitotic divisions in Colchicum are immune to colchicine up to 1%, but they are affected by acenaphthene (Levan 1940).

Certain growth substances, e.g. naphthalene-acetic acid, have been reported by Dermen (1941) and Levan (1939a) to have induced intranuclear polyploidy or root swelling. As mentioned above, Greenleaf (1938) used one of these substances to induce greater callus formation in Nicotiana, and thus at least an indirect effect.

Other chemicals have been used successfully in producing polyploids. Sodium cacodylate was effective on Portulaca but not on Datura (Blakeslee 1939). Tetraploid snapdragons were produced by treatment with sanguinarine from bloodroot (Little 1942).

Many other substances have been found to have a polyploidizing effect on plant cells, but in most cases progeny have not been grown. A few of the numerous references are: Kisser and Lindenberg (1940), Gavaudan and Gavaudan (1940), Gosselin (1940), mercury seed treatment compounds, Mechelke (1958), and Oehlkers and Linnert (1951).

For a discussion of synergists and antagonists to the action of colchicine, see Eigsti and Dustin (1955).