OFF-FLAVOUR DEVELOPMENT IN FROZEN GREEN BEANS

by

David H. Lees

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Lees

ABSTRACT

M.Sc. DAVID H. LEES Horticulture

THE DEVELOPMENT OF OFF-FLAVOUR IN FROZEN GREEN BEANS

An investigation was undertaken to study the correlation which may exist between off-flavour development, determined organoleptically, and lipid oxidation, as determined by means of the 2-thiobarbituric acid test in frozen green beans. A procedure for the extraction of the 2-thiobarbituric acid reactive substance of beans was developed. Satisfactory and reproducible results were obtained with the method employed.

Raw and blanched beans were stored under oxygen, nitrogen and air at -9.4°, -15.0° and -20.6°C for varying periods of time to obtain information on the mechanism of off-flavour development. The 2-thiobarbituric acid test was carried out at regular intervals throughout the storage period and organoleptic tests were conducted concurrently. A satisfactory relationship was found to exist between the 2-thiobarbituric acid numbers and the development of off-flavour in frozen green beans. The use of the 2-thiobarbituric acid test to detect the development of off-flavour in frozen green beans is proposed.

Efforts were made to identify the carbonyl compounds of beans by means of their 2,4-dinitrophenylhydrazine derivatives using thin-layer chromotography and ultraviolet absorption studies.

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INTRODUCTION

It is well recognized in the commercial preservation of vegetables by freezing that certain enzymatic changes can cause deterioration at low temperatures. These changes are generally evidenced by the development of unnatural, disagreeable odours and flavours, and discolouration. That off-flavour occurred in raw or underblanched vegetables as a result of an imbalance in the enzyme reactions was recognized early in the development of the frozen vegetable industry by Joslyn (1930), Tressler (1932) and Diehl and Berry (1933). Numerous investigations have been carried out on various aspects of this problem but the actual reactions involved are still largely unknown. The investigators agree that the off-flavours are due to an enzymatic process since they do not develop in properly blanched vegetables. However, on prolonged storage non-enzymatic changes resulting in disagreeable flavours are probably also involved.

The enzyme systems directly or indirectly responsible for quality deterioration in raw or under-blanched vegetables freezing storage are as yet unknown;

however, those commonly associated with quality changes during frozen storage are lipase, lipoxidase, catalase and peroxidase (Lee and Wagenknecht, 1958). Besides the enzymes actually involved in off-flavour development investigations are being carried out to determine the substrates acted upon, the factors influencing the rate and extent of chemical change, and the chemical components responsible for off-flavour formation. It has been shown that lipoxidase and lipase are responsible for the progressive deterioration of lipids in raw vegetables during frozen storage (Lee and Wagenknecht, 1951; and Wagenknecht and Lee, 1956). The changes in the lipids closely parallelled the progressive development of off-flavours and there was reasonable evidence that lipid oxidation was one of the, if not the, major factor responsible for off-flavour production in frozen raw vegetables (Lee et al., 1955; Lee and Wagenknecht, 1951). The changes in flavour occurring in frozen, raw or underblanched vegetables have also been attributed to autolysis and proteolysis (Tressler, 1932; Mergentime and Wiegand, 1946) and glycolysis (Joslyn, 1949).

The present investigation was undertaken to study the correlation which may exist between off-flavour development, which was judged organoleptically,

and lipid oxidation, as determined by means of the 2-thiobarbituric acid test (TBA). This test has been applied successfully for detecting fat oxidation in a number of materials including dairy products (Patton and Kurtz, 1951; Dunkley, 1951; Biggs and Bryant, 1953), pork (Turner et al., 1954), bakery products (Caldwell and Grogg, 1955), oysters (Schwartz and Watts, 1957), fishery products (Yu and Sinnhuber, 1957; Munker, 1962), and other biological materials. A procedure for the application of the test was developed which gave satisfactory and reproducible results.

The present investigation was carried out on green snap beans for which relatively little information is available. Raw and blanched beans were stored under oxygen, nitrogen and air to obtain information on the mechanism of off-flavour development. Samples were stored at three different temperatures and the TBA test was carried out at regular intervals throughout the storage period and organoleptic tests were carried out simultaneously. A satisfactory correlation was found to exist between the TBA values and the organoleptic scores for the development of off-flavour in frozen beans.

During this investigation it was found that malonaldehyde, which shows an absorption maximum of

245 mμ at pH < 3, could not be detected in any of the bean samples. This is in agreement with the results of Kwon and Watts (1963) who reported that the ultraviolet absorption spectra of distillates from peas were complex and different in appearance from that of MA. A condensation product between TBA and one or more of the carbonyl compounds in beans could be responsible for the colour formation which shows maximum absorption at 535 mμ, since the TBA reagent is not specific for MA (Patton, 1960). This was also shown by Saslaw and Waravdekar (1965), who demonstrated, by thin-layer chromatography of irradiated fatty acids, that none of the thiobarbituric acid reactive substance (TBRS) was MA.

A large number of carbonyl compounds are present in beans, as demonstrated in this investigation by the many 2,4-dinitrophenylhydrazine (2,4-DNPHine) derivatives obtained. Efforts were made to identify these carbonyl compounds by means of thin-layer chromatography and their ultraviolet absorption spectra.

I. REVIEW OF LITERATURE

The Role of Enzymes in Off-flavour Development

That changes occur in the flavour of most underblanched vegetables during freezing storage has been reported by numerous workers (Joslyn, 1946, 1949, 1961; Mergentime and Wiegand, 1946; Woodroof *et al.*, 1946; Lee *et al.*, 1955; Dietrich *et al.*, 1962). These changes have been ascribed to the activity of naturally occurring enzymes not inhibited by low temperatures and ice formation. Makower (1956) reported that enzymes are specific biochemical catalysts present in all living tissues. They affect the quality of processed foods by producing chemical changes in vegetables and fruits before, during, and sometimes after processing, resulting in poor flavour, texture and colour. The undesirable off-flavours produced during freezing storage of raw and underblanched frozen vegetables are believed to be due to the accumulation of unknown rather volatile compounds (Joslyn, 1949).

The four enzymes normally associated with off-flavour development are catalase, peroxidase, lipase and

lipoxidase. Extensive research has been carried out on these enzymes. The pertinent literature on these enzymes is presented in this section, placing emphasis on the data available on beans and other vegetables.

It is well established that certain enzymes or enzyme systems must be inactivated or partially inactivated if vegetables are to retain their quality during freezing storage. This inactivation is usually accomplished by blanching. Since the enzyme or enzyme systems directly responsible for quality deterioration in raw or underblanched vegetables during freezing storage are as yet unknown, the activity of certain enzymes, such as peroxidase and catalase, has been used to determine the adequacy of blanching.

Diehl and Berry (1933) and Diehl et al. (1933) stated that catalase activity could serve as an index of adequacy of blanching for peas and indicated that Alderman peas blanched for a time sufficient to destroy catalase activity retained satisfactory quality when stored at -6.5°C or lower. The use of catalase as a measure of adequate blanching for the freezing storage of vegetables was also recommended by Tressler (1933), Diehl et al. (1936) and Kramer (1954). Lineweaver and Morris (1947) proposed a test for catalase activity and

reported that if 0.5% of the original catalase content was still present, blanching was insufficient. Arighi et al. (1936) observed that catalase was quantitatively inactivated at a much more rapid rate than the enzymes concerned in the production of off-flavours in peas and suggested that the enzymes concerned in the production of off-flavours in peas were more heat stable than catalase.

Tressler (1939) reported that, in general, vegetables blanched sufficiently to inactivate catalase and peroxidase would not develop off-flavour during freezing storage. He indicated that peroxidase appeared to be the more heat stable enzyme, therefore making it more suitable for testing for adequacy of blanching. Bedford and Joslyn (1939), working with frozen string beans, concluded that peroxidase activity was a better index of quality than catalase activity. They reported that peroxidase activity need not be completely destroyed to obtain a satisfactory retention of flavour. These findings were supported by Joslyn and Bedford (1940), who found that flavour retention in frozen asparagus was closely related to the inactivation of peroxidase by blanching.

Balls (1942, 1947) and Makower (1956) suggested

that peroxidase activity might serve as an index of adequacy of blanching. In a review paper, Joslyn (1949) referred to earlier work in which it was found that peroxidase activity more closely parallelled the formation of off-flavours in frozen vegetables than did catalase activity. However, he cautions that the correlation varied markedly with the nature of the substrate used for detecting peroxidase activity. Masure and Campbell (1944), in a quantitative study of the degree of peroxidase inactivation and quality retention in frozen vegetables, showed that samples heated long enough to insure quality retention during subsequent freezing storage contained 1 to 2% residual peroxidase. However, Kramer and Mahoney (1940) reported that the semiquantitative determination of peroxidase is unsatisfactory as an index of quality for frozen lima beans. Methods of determining peroxidase activity and their usefulness have been proposed by Joslyn (1957) and Joslyn and Neumann (1963).

Lindquist et al. (1951) stated that a blanching treatment adequate to inactivate catalase was insufficient for the retention of quality in Brussels sprouts. However, peroxidase activity was found to serve as a satisfactory index of adequacy of blanching. Samples

blanched sufficiently to inactivate catalase but not peroxidase showed a marked development of off-flavour, a decrease in natural flavour and the development of pink colouration at the centres within a short time after processing, when compared with peroxidase-negative samples.

Blanching to a point sufficient for catalase inactivation was shown by Dietrich et al. (1955) to be insufficient for flavour retention, but sufficient for chlorophyll and ascorbic acid retention, when stored under good commercial conditions of -17.8° to -23.3°C. However, samples blanched long enough for partial peroxidase inactivation were better in flavour and colour and retained a greater amount of ascorbic acid than samples blanched for longer or shorter times. These findings are in agreement with those of Joslyn (1946).

Beale and Gould (1959) carried out studies on the effect of the length of the blanching period on the peroxidase activity of frozen corn-on-the-cob. They proposed that a satisfactory product may contain a small amount of residual peroxidase activity, which may be preferred to overblanching of the product. However, Zoneil and Esselen (1959), studying the thermal

destruction rates and regeneration of peroxidase in green beans and turnips, stated that if all the peroxidase is not destroyed in vegetable tissue, it could slowly regenerate, even under frozen storage conditions, and eventually cause a disagreeable flavour in the vegetables. Boettcher (1962) found that in frozen green peas, beans, cauliflower, Brussels sprouts, kale, spinach, sliced carrots, and asparagus, the higher the peroxidase activity after blanching the poorer the quality of the vegetable after freezing preservation.

Boettcher (1962) reported the regeneration of peroxidase activity in green peas and beans blanched in water at temperatures varying from 75° to 100°C after 6 to 9 months of storage. However, the regeneration was small and did not appear to have any significance since the organoleptic quality of the samples was not impaired, and it occurred only in samples pre-heated at temperatures below those necessary for blanching. Similar results have been reported by Diehl et al. (1936) and Woodroof et al. (1946). Pinsent (1961) found a regeneration of peroxidase at -18°C. Joslyn (1961) reported the regeneration of peroxidase activity at room temperature.

With the establishment of the necessity to inactivate enzymes by heat prior to freezing, the

development of the proper conditions for blanching and its application to various vegetables was investigated. The storage life of blanched vegetables at various temperatures was also studied.

Joslyn and Marsh (1933) reported that peas, spinach and string beans each had a critical blanching temperature range which favoured better colour, flavour and texture retention. These were: for peas 71°-76.5°C, for spinach 73.9°-82.2°C, and for string beans 82.2°-90.6°C. They found that, when underblanched, these vegetables developed unnatural hay-like flavours in two months at -17.5°C when exposed to air. The discolouration of artichoke hearts was studied and found to be related to the extent of blanching (Joslyn and Marsh, 1938). Asparagus was reported to have optimum flavour retention when blanched at 92°C for 4 minutes or 100°C for 3 minutes (Joslyn and Bedford, 1940). Other investigations relating blanching practice and storage temperature to quality retention were also carried out by Woodroof (1931), Tressler (1932, 1939), Diehl and Berry (1933), Diehl et al. (1936), Heiss and Peach (1937), Woodroof et al. (1946), Cruess (1947), Gortner et al. (1948), Lindquist et al. (1950), Fisher and van Duyne (1952), Noble and Winter (1952).

Dietrich et al. (1959, 1962), in a series of investigations, dealt with the time-temperature tolerance of frozen beans, cauliflower and spinach. The rate of deterioration in flavour and colour of frozen beans approximately doubled for each 2.7°C increase in temperature between -17.8° and -3.6°C. In cauliflower the rate of deterioration increased about 4-fold for each 5.5°C raise in temperature in the range of -17.8° to -3.6°C. The cauliflower was acceptable but not of high quality after 3 to 6 months storage at -12.2°C and 5 to 11 weeks at -6.6°C. In spinach, deterioration increased as the temperature increased. They concluded that low storage temperatures are essential for the preservation of quality of these frozen vegetables.

Joslyn (1949), in a review on enzyme activity in frozen vegetable tissue, stated that "enzymes are responsible for the development of off-flavour in raw and underblanched vegetables during frozen storage." He reported that as the storage temperature decreases the rate of development of off-flavours decreases, and that scalding and subsequent chilling should be conducted under conditions such that enzymes responsible for off-flavour development during storage will be inactivated with minimum effect on flavour and texture.

The time and temperature of blanching are dependent on heat transfer and other biological factors of the material being treated.

The most likely cause of off-flavour development in underblanched or unblanched frozen vegetables is the enzyme-catalyzed oxidative deterioration of lipids. The crude lipid content of vegetable tissues is very small, varying from 0.1 to over 1% on a fresh weight basis, depending on the vegetable (Joslyn, 1961). Siddiqi and Tappel (1956) reported that the activation energy for lipoxidase was 4.3 K cal/g mole and the activation energy of autoxidation was 15.2 K cal/g mole. Therefore, the enzymatic oxidation of lipids could be very important, and the damage caused by autoxidation of the lipid material would be negligible in comparison with that caused by enzymatic oxidation at low temperatures of storage.

Lipoxidase was first described by André and Hoy (1932) who isolated it in an oil extract of soybean residue. André and Hoy (1933) further demonstrated its presence in Hyacinth beans and common kidney beans and established it as being separate from peroxidase. The presence and determination of lipoxidase in other vegetables has been reported for asparagus (Sumner, 1943)

and green and dry garden peas (Strain, 1941).

The action of lipoxidase in converting unsaturated fats into peroxides was reported by Strain (1941). He stated that only those compounds with the $-CH=CH(CH_2)_7CO-$ group and cis-configuration, e.g., oleic, ricinoleic, linoleic, linolenic acids and their esters, were oxidized directly. Sumner (1942) further explained the action of lipoxidase and concluded that lipoxidase was stereo-specific showing a specificity for the cis-configuration of the double bond in the 9-10 position, and acted most efficiently on the structure $RCH=CHCH_2CH=CH(CH_2)_7COOH$. The presence of an additional isolated double bond increased the rate and extent of peroxidation. However, the presence of more than 2 double bonds merely served to increase the activity of peroxidase without increase in peroxidation. The oxidation of the more unsaturated fatty acids by lipoxidase was found to be very rapid by Sullmann (1943). Lipoxidase has been reported to be highly specific for the oxidation of fatty acids liberated by lipolytic enzymes (Balls et al., 1943; Oser, 1946; Siddiqi and Tappel, 1956).

Lipoxidase is reported to have an optimum temperature for activity of about 30°C and a Q_{10} of about 1.6 between -1.5° and 18.5°C. Increased

temperature and/or pH have a deleterious effect on the activity of lipoxidase (Holman, 1947; Smith, 1948; Asada, 1952). Lund and Halvorson (1951) demonstrated that lipoxidase can function at low temperatures and that lipid deterioration in foods containing lipoxidase cannot be adequately prevented by storage at low temperatures as long as the reactants are allowed to come in contact through a liquid medium. This activity is greatly reduced, however, when such systems are completely solidified since the diffusion rates become the limiting factors.

The other enzyme involved in the deterioration of the lipid fraction in frozen vegetables, is lipase. Its action in frozen material was demonstrated as early as 1900 (Kastle and Loevenhart, 1900) and Street and Bailey (1915) indicated its presence in soybeans.

Armstrong and Omerod (1907) found that the hydrolysis of esters by lipase involved the direct association of the enzyme with the carboxyl centre (CO.O) and that hydration of this centre prevented this action. The deterioration of fats in frozen storage at temperatures as low as -30°C has been attributed to the hydrolysis of the fats by lipase (Balls and Tucker, 1938; Balls and Lineweaver, 1938). These workers also

reported that the specificity of lipase appears to be temperature dependent, since the velocity of the enzyme reaction is greatly decreased with the change of state from liquid to solid, and different fats and esters are split at very different rates, the most rapid being the glycerides of the lower fatty acids and those with 6 to 10 carbon atoms. These findings have been substantiated by Lineweaver (1939), Balls (1940) and Kuprianoff (1961). Lipase has been found to be active at temperatures as low as -70°C (Sizer and Josephson, 1942).

The hydrolysis of fat in frozen tissues, by lipase, presents therefore a definite and detectable change in chemical composition which has an important bearing on the quality of the stored product.

Lee and Wagenknecht (1951) found that substantial changes occurred when unblanched Thomas Laxton peas, stored at -17.8°C for five years, were compared with blanched and unblanched peas of the same variety immediately after harvesting. These changes were observed in the crude lipids of peas obtained by extraction with peroxide-free anhydrous ethyl ether. As a result of this investigation, in which the peroxide number, acid number, iodine number and odour and taste tests were used, they concluded that developing rancidity

of the lipid material was one of the prime causes, if not the principal cause, of the development of off-flavour in unblanched frozen peas stored at -17.8°C. It was proposed that lipoxidase and lipase are responsible for the progressive deterioration of lipids in raw peas during frozen storage.

These researchers also reported that a considerable loss of chlorophyll and carotene occurred during the protracted storage of frozen unblanched peas. However, no significant changes were observed in the reducing and non-reducing sugar fractions and in the starch fraction. Thus it appeared that these components of the carbohydrate fraction did not contribute to the development of off-flavour in the frozen unblanched peas. In addition, no changes were observed in total nitrogen content, such as would have occurred had ammonia, or volatile amines, or other volatile nitrogenous compounds been evolved during frozen storage.

Wagenknecht (1952), working on frozen raw peas, reported that marked changes occurred in the lipid fraction. He considered these changes as being mainly responsible for the off-flavour and odour of the peas frozen for an extended period of time (five years at -17.8°C). In raw peas, frozen and stored for 5 years,

a loss of more than 50% of the chlorophyll and carotene was observed. However, no changes in proteins, starch, and sugar fractions occurred. Blanched peas stored under identical conditions showed very little loss of chlorophyll.

Lee (1954) conducted a study on the crude lipids extracted from asparagus, corn, Lima beans, snap beans, spinach and peas, after frozen storage. As a result of this study, he proposed that enzymatic oxidation of lipid matter is the actual cause of off-flavour in these frozen vegetables, since the acid numbers increased after 1 week and peroxides appeared after 1 month of storage at -17.8°C.

Lee et al. (1955) undertook a study to determine the length of time unblanched vegetables could be held in frozen storage before undesirable changes could be detected, and to determine the cause of the development of the acids and peroxides during the storage period. Unblanched peas, corn and snap beans were investigated. The results indicated that definite off-flavour could be detected in all three vegetables after 2 to 4 weeks of storage and also that peroxides could be detected in peas after 3 weeks, in snap beans after 1 month, and in corn after 2 months. It was suggested that, since

off-flavours increased in intensity in the raw vegetables with increasing storage time, and since peroxides started to accumulate later than acids, a sequence of enzyme actions was involved in the development of the more disagreeable off-flavours and off-aromas of the unblanched vegetables during extended storage. The milder off-flavours were said to be brought about, at least partially, by an increase in acid. They reported that the changes in lipids, characterized by increases in titratable acidity and the development of peroxides, and the loss of chlorophyll, closely parallelled the progressive development of off-flavour.

The presence of peroxides in blanched sweet corn after one and one-half years was also reported by Lee et al. (1955). This was first reported by Lee (1954) and he suggested that it occurred as the result of non-enzymatic peroxidation.

Wagenknecht and Lee (1956) established the presence of lipoxidase in fresh and frozen raw peas. They obtained the enzyme in partially purified form from fresh and lyophilized raw peas. They demonstrated that the two major actions of lipoxidase in peas were peroxidation of lipids and destruction of chlorophyll, and proposed reactions through which lipoxidase may

contribute to off-flavour formation and other changes in quality of raw peas.

Siddiqi and Tappel (1956) reported that pea extracts contained an enzyme that behaves like a true lipoxidase, in that it did not possess fatty acid oxidase or fatty acid dehydrogenase activity, and yet it oxidized linoleate but not oleate. They proposed that, in the intact pea, lipoxidase may react with the unsaturated fatty acids present to form hydroperoxides which in turn decompose to give aldehydic compounds. Thus lipoxidase could cause off-flavour by lipid oxidation, and this in turn could be used to explain the accumulation of aldehyde as off-flavour develops.

Lee (1958) stated that lipoxidase and lipase are responsible for the development of off-flavours in raw and underblanched vegetables during frozen storage at -18°C. In reviewing the practice of adequate blanching for the inactivation of catalase and peroxidase enzymes, he proposed the use of the time required for catalase inactivation plus 50% of this time as a safety factor for good results. He also found that rancidification of the lipid material, as evidenced by increases in the peroxide and acid numbers of the extracted lipids, had a great deal to do with increases in off-flavour, the

effect increasing with longer storage times.

Lipoxidase, lipase, peroxidase and catalase, the four enzymes thought to be associated with quality changes during frozen storage, were studied by Lee and Wagenknecht (1958). The enzymes were obtained in partially purified form from lyophilized raw peas. Addition of these partially purified preparations of native pea enzymes to blanched peas resulted in the production of off-flavours, following prolonged frozen storage. The greatest flavour change was associated with added catalase, but lipoxidase and lipase produced pronounced off-flavour. Peroxidase produced only a mild change in flavour which was not objectionable. Changes in green colour were brought about by the addition of all four enzymes, with the greatest change being produced by lipoxidase and lipase. The deterioration of lipids was also evidenced by the formation of peroxides and by moderate increases in acid number. As a result of their findings they concluded that it seems likely that off-flavour production in frozen raw vegetables is quite complex in nature and that the concerted action of several or many enzymes is involved.

Earlier work by Wagenknecht and Lee (1958) using model systems to study the action of catalase, peroxidase,

lipase and lipoxidase had shown that the addition of these enzymes to blanched peas resulted in off-flavours following frozen storage. The lipase-treated peas gave the most disagreeable flavours, while lipoxidase, catalase and peroxidase produced only mild flavour changes. Changes in green colour were produced by the addition of all four enzymes. Lipase caused the greatest losses of chlorophyll, evidenced by the formation of pheophytin. The deterioration of lipids was caused by lipase and lipoxidase and resulted in large increases in the acid number and the development of peroxides. As a possible mode of action of lipase, they proposed that it may serve as an adjunct to lipoxidase in off-flavour development through a labilization of substrates, i.e., the lipase splits the triglycerides into free fatty acids which are apparently more readily utilizable as lipoxidase substrates than the intact triglycerides. During the progressive development of off-flavour in frozen raw peas during storage, the increase in acid number invariably preceded the development of peroxides detectable in the lipids.

The carbonyl content in the crude lipid of peas, prepared from blanched and unblanched peas, held in frozen storage at -17.8^{o}C for 5 years, and from fresh

peas, was studied by Lee (1958). The stored unblanched peas yielded crude lipids which contained appreciable amounts of unsaturated carbonyl compounds. Unsaturated carbonyl compounds were not found in the crude lipids extracted from material blanched prior to storage, or in fresh material, either raw or blanched. However, the crude lipids extracted from the blanched peas contained more saturated carbonyl compounds than that extracted from the corresponding raw peas, fresh or stored. From these results, it was thought that a possible connection between the development of peroxides and the formation of unsaturated carbonyl compounds might exist.

The fatty acids of peas and spinach were investigated by Lee and Mattick (1960). They found significant differences between raw and blanched samples when stored at -17.8°C for extended periods of time. In peas, the raw samples showed large losses in the fatty acids from the neutral fats, when compared with blanched samples. In spinach, it was determined that the total free fatty acids increased in the raw samples during storage. Palmitic acid increased during storage, whereas the longer chain fatty acids, particularly linolenic acid, decreased.

Wagenknecht (1963), in a study on green beans, found that they contained 2.6% lipids on a dry weight basis. Varying amounts of chlorophyll and its degradation products were present in all lipid fractions and complete removal of these chlorophyll pigments was not achieved.

The glyceride fractions of Lima beans and four other types of beans were reported to consist mainly of palmitic, linoleic and linolenic acids, together with smaller amounts of stearic and oleic acids (Korytnyk and Metzler, 1963). The Lima bean lipid fraction included n-pentadecanoic and n-heptadecanoic acids. A very high proportion of linolenic acid, associated with a high iodine value, was noted in all beans investigated. An unusual characteristic of the bean lipids was the almost complete absence of higher saturated fatty acids. Fatty acids with an odd number of carbon atoms were also found in beans.

Lundberg (1956) stated that the main products of the lipoxidase-catalyzed oxidation of polyunsaturated fatty acids were optically active cis-trans conjugated diene hydroperoxides. He also stated that the lipoxidase oxidation may involve some type of chain oxidation which must differ from that involved in autoxidation.

Loury (1963) suggested that, in autoxidative rancidification of fats, the peracid formed cleaves into the next lower aldehyde and HCO_2H which further oxidizes to CO_2 and H_2O. The new aldehyde is then oxidized to peracid, which undergoes the same cycle.

Lipid hydrolysis in unblanched frozen peas, in the range of -5° to $-20^{\circ}C$, was reported on by Bengtsson and Bosund (1966). The Q_{10} value for the formation of free fatty acids in this temperature range was about 2.5 and the corresponding value for off-flavour development was 3.0. At the lower temperature range a definite preference for hydrolysis of polyunsaturated acids was reported, with a corresponding increase in the proportion of saturated acids in the unhydrolyzed fat. Changes in the lipid fraction of vegetables were shown to contribute to, and possibly be one of the major components of, off-flavour development in raw frozen peas.

Acetaldehyde and Alcohol in Plant Tissues

The accumulation of volatile aldehydes in the tissues of frozen raw and underblanched beans was first reported by Bedford and Joslyn (1939). They found that as the duration or temperature of blanching increased the acetaldehyde content decreased and the quality of

the product improved. Joslyn (1949), in a review paper, pointed out that glycolytic changes leading to the accumulation of acetaldehyde and alcohol were related to enzyme activity and off-flavour formation in frozen unblanched and underblanched vegetables.

The correlation between off-flavour development and production of acetaldehyde and alcohol was thoroughly investigated with peas (Arighi et al., 1936; Gutterman et al., 1951; Moore, 1951; Joslyn and David, 1952; Lovejoy, 1952; David and Joslyn, 1953; Kramer, 1954; Wager, 1958; Ralls, 1960; Matthews, 1961; Pendlington, 1962; Self et al., 1963; Bengtsson and Bosund, 1964). Acetaldehyde was proposed as an objective measurement of off-flavour for frozen peas (Arighi et al., 1936; Gutterman et al., 1951; Lovejoy, 1952; David and Joslyn, 1953), Lima beans (Kohman and Sanborn, 1934), artichoke hearts (Joslyn et al., 1938), and asparagus (Joslyn and Bedford, 1940; Lovejoy, 1952), and it was adopted by the AOAC (1955) as the official method for peas and asparagus. However, other investigators found the correlation unsatisfactory (Moore, 1951; Kramer, 1954; Fuleki, 1961; Smith, 1966).

Alcohol content, rather than aldehyde, was found to be in good correlation with off-flavour in broccoli (Buck and Joslyn, 1953).

Fuleki (1961) studied the effects of blanching, storage temperature, and container atmosphere on acetaldehyde and alcohol production and off-flavour formation in frozen green snap beans. He found that aldehydes, determined as acetaldehyde but not identified as such, accumulated in green snap beans but that its accumulation could not be suggested as an objective measure of off-flavour development. He also reported that the amount of acetaldehyde which accumulated over the initial content depended on three main factors: extent of blanching, length of storage and temperature of storage. Fuleki found also that the ethanol content of green beans was decreased by blanching but that raw or lightly blanched beans retained their ability to produce alcohol. The factors affecting alcohol accumulation are the same as those reported for acetaldehyde accumulation. Fuleki concluded that acetaldehyde and alcohol accumulation did not provide an objective measurement of off-flavour development in frozen green snap beans.

Smith (1966) found that no relationship could be established between aldehyde and ethanol production and off-flavour development in frozen raw cauliflower. He concluded that neither aldehyde nor ethanol content provided a satisfactory objective method for the determination of off-flavour development.

Use of TBA in the Study of Oxidative Rancidity

That a reaction occurred between TBA and a number of aliphatic aldehydes, to form highly coloured derivatives, was first reported by Dox and Plaisance (1916). Kohn and Liversedge (1944) observed that various animal tissues, after incubation under aerobic conditions, produced compound(s) which gave a colour reaction when heated with TBA. Although they did not demonstrate the nature of the colour producing compound(s), their research established the absorption spectrum of the colour complex and certain chemical characteristics of the colour reaction. They reported that the responsible compound(s) were carbonyl in nature, since semicarbazide and phenylhydrazine blocked the reaction. Research by Bernheim *et al.* (1948) revealed that the colour-producing materials in various animal tissues were lipid in nature and, more specifically, that egg lecithin or fatty acids isolated from it, brain lecithin, fatty acids from linseed oil, cephalin, and brain protein-lecithin complex, gave colours with TBA after aerobic incubation with ascorbic acid. The absorption spectra of the colours obtained from these materials were shown to be identical with that described by Kohn and Liversedge (1944) and that obtained with linolenic acid. They also suggested

that a three-carbon compound containing one oxygen is involved in the TBA reaction to form the coloured pigment. Wilbur et al. (1949) further explored the TBA colour reaction with regard to certain sugars and aldehydes, as well as to the oxidation products of linolenic and certain other unsaturated fatty acids. They could not find any specific compound giving a colour spectrum identical to those obtained from oxidized lipid materials in aerobically incubated animal tissues, the latter showing a single peak at 532 mμ, characteristic of the colour from oxidized lipid materials.

Patton and Kurtz (1951) reacted a number of compounds with TBA, in addition to those investigated by Wilbur et al. (1949). They also developed a suitable method for the application of the TBA test in the measurement of milk fat oxidation. They showed that malonic dialdehyde yielded a red colour when heated with TBA reagent. Spectral analysis of this colour revealed it to be identical with that obtained with oxidized milk fat, and to resemble closely colours secured in like manner from a number of oxidized lipid materials and animal tissues containing unsaturated fatty acids such as oxidized methyl linolenate (Wilbur et al., 1949), oxidized phospholipids (Bernheim et al., 1948), and

aerobically incubated animal tissues (Kohn and Liversedge, 1944; Wilbur et al., 1949). They concluded that malonic dialdehyde may be a compound of significance in food fat rancidification and in the biological oxidation of unsaturated fatty acids. They also proposed that the TBA test could be used advantageously in measuring oxidative deterioration in a wide variety of fats and fat containing foods.

A good correlation between the TBA test and numerical flavour scores of milk samples, having oxidized flavour of varying intensity, was reported by Dunkley (1951). He also reported that the TBA test was, in fact, slightly more accurate in determining the percentage of oxidized milk. Dunkley and Jennings (1951) presented a procedure for the application of the TBA test to oxidized fluid milk.

The TBA reaction has been studied as a measure of oxidative rancidity in a wide variety of food products by a large number of investigators. Biggs and Bryant (1953) modified the TBA test to detect oxidative rancidity in cheese, butter, and whole milk powder and concluded that this test was more sensitive than conventional tests such as iodine value and Kries test. Tanaka et al. (1955) found that the TBA test satisfactorily

established the intensity of oxidized flavour in whole dried milk. Sidwell et al. (1955) applied the TBA test to dried milk products. They employed steam distillation to separate the products of fat oxidation from dried milk products in order to avoid interference from the lactose and protein constituents. Their results correlated well with sensory evaluations.

Turner et al. (1954) described a method using TBA for the determination of rancidity in frozen pork. They reported that the TBA test gave a more reliable index of age and quality of frozen pork than other chemical tests. Zipser et al. (1964) also reported favourable results with the use of TBA with cured and uncured frozen cooked pork.

The application of the TBA test as a measure of fat oxidation in vegetable oils and lard has been reported by numerous investigators (Sidwell et al., 1954; Romero and Gonzalez-Quijano, 1956; Schmidt, 1959; Khomutov and Garkusha, 1960; Szilas-Kelemen and Bereczky, 1962; Mihelic, 1963, 1964; Pohle et al., 1964; Wyatt and Day, 1965).

Yu and Sinnhuber (1957) proposed a TBA method for the measurement of rancidity in fishery products. They performed the TBA test on the intact sample eliminating

the fat extraction step which is often times difficult, incomplete, and time-consuming. Their procedure was simple and gave reproducible results on fish oil, fish meal, and canned and frozen fishery products. Their results indicate that it can be used as a measure of oxidative rancidity in fishery products. Sinnhuber and Yu (1958) later suggested a TBA method for the measurement of rancidity in fishery products, using 1,1,3,3-tetraethoxypropane (TEP) as a standard for the TBA determination of malonaldehyde (MA), by which the MA content of fishery products could be quantitatively measured, and the degree of oxidative rancidity expressed in milligrams of MA per 1000 g of sample. Other researchers on fishery products include Schwartz and Watts (1957), Palmeter et al. (1960), and Munker (1962).

Caldwell and Grogg (1955) found the TBA test in cereal and baked products to provide a more sensitive and reproducible means of detecting and recording incipient oxidative rancidity than the peroxide value technique.

The TBA test has also been used in the measurement of oxidative rancidity of cooked stored meats (Fujimaki and Yoshimatsu, 1960); stability of ascorbic acid solutions (Smoczkiewiczowa and Grochmalicka, 1961);

cooked mullet (Zipser and Watts, 1961); roasted Macadamia nuts (Winterton, 1962); fresh pork, ham, meat, bread, potato chips, and lard (Tarladgis et al., 1964); milk, meat, fats and oils, fish, soups, cereals, baked goods, and walnut kernels (Purr, 1964). Tarladgis and Watts (1960) employed the TBA test to study MA production during the oxidation of pure unsaturated fatty acids under controlled conditions.

In this laboratory, Smith (1966) used the TBA test as a means of following the development of off-flavour in frozen raw cauliflower and found a satisfactory correlation between TBA values and organoleptic testing of off-flavour.

Efforts to characterize the TBA pigment have been reported by several investigators. Patton et al. (1951) pointed out the similarity between epihydrin aldehyde and malonaldehyde. They reported that the TBA reactive material from oxidized milk fat was a water soluble, low molecular weight, Kreis positive, carbonyl compound similar to malonaldehyde. In addition, they postulated that malonaldehyde would be strongly acidic, enolic, and relatively stable upon heating with dilute mineral acids.

Kurtz et al. (1951) tentatively identified the compound in oxidized milk fat which reacts with TBA as

malonaldehyde and postulated that the reaction occurred between the monoenolic form of MA and the active methylene group of TBA, followed by ring closure.

Using a spectrophotometric technique, Jennings et al. (1955) suggested that the MA-TBA pigment was formed by an equi-molar reaction without the loss of water. They reported that, although the pigments prepared from TBA and oxidized milk fat, MA and 2-sulfanilamidopyridine, exhibited the same spectral characteristics in the visible range, this did not justify the conclusion that they were identical.

Sinnhuber et al. (1958) prepared the pure pigment from both malonaldehyde and rancid oil (salmon oil). On the basis of elemental analyses, absorption spectrophotometry, and paper chromatography, they concluded that pigments from the two sources were identical and suggested a structure in which two molecules of TBA condense with one of malonaldehyde. This structure was confirmed by Schmidt (1959) (as quoted by Brownley and Lachman, 1965).

More recently, Taufel and Zimmerman (1960, 1961) published results on the chemistry of the colour reaction between TBA and carbonyl compounds. They found that the formation of malonic dialdehyde during oxidation is not

confined to the nonconjugated unsaturated fatty acids, but occurs with saturated and conjugated acids. They also reported that the reaction of one molecule of TBA with one molecule of aldehyde gives a colourless compound which is analogous to an aldol. This could further substantiate the reaction proposed by Sinnhuber et al. (1958).

The application of the TBA test to food products has varied widely, with several modifications being proposed by various investigators.

Individual methods of performing the TBA test have been outlined by several investigators (Bernheim et al., 1948; Wilbur et al., 1949; Dunkley, 1951; Dunkley and Jennings, 1951; Patton and Kurtz, 1951; Biggs and Bryant, 1953; Sidwell et al., 1954; Turner et al., 1954; Caldwell and Grogg, 1955; Sidwell et al., 1955; Tanaka et al., 1955; Romero and Gonzalez-Quijano, 1956; Schwartz and Watts, 1957; Yu and Sinnhuber, 1957; Sinnhuber and Yu, 1958; Sinnhuber et al., 1958; Schmidt, 1959; Palmeter et al., 1960; Tarladgis et al., 1960; Munker, 1962; Zipser and Watts, 1962; Tarladgis et al., 1962; Purr, 1964; Brownley and Lachman, 1965; Smith, 1966).

Tarladgis et al. (1962) classified the method

under two categories: (a) A solution of TBA in a strong acid is added to the food product, and the whole mixture is heated for periods of 10 to 35 minutes in a water bath to obtain maximum colour development. The red pigment is then extracted with a suitable solvent and measured in a spectrophotometer; (b) The food product is first steam distilled with acid, and the TBA acid solution is added to a portion of the distillate, which is then heated for 35 minutes for maximum colour development. The red pigment is measured directly in a spectrophotometer.

All the proposed methods for performing the TBA test invariably employ the addition of TBA dissolved in acid. The two methods outlined by Tarladgis et al. (1962) are similar in that they both employ heating of the food at a low pH which is claimed to be essential for the liberation of MA from some precursor, as well as for its condensation with TBA. The differences in the methods employed, however, are many.

Tarladgis et al. (1960) proposed a distillation method for the quantitative determination of MA in rancid foods using TBA. Their distillation method appears to have advantages in that prolonged heating of the food product is avoided, thus keeping to a minimum any further

oxidative or decomposition changes during the test. The acid-heat treatment necessary to effect the liberation and distillation of MA from the sample is less drastic than that required for maximum colour development with the TBA reagent. Furthermore, only the volatile constituents of the food are distilled over, thus avoiding any possible reaction of the TBA with nonvolatiles in the food. In addition, the acid in the TBA reagent is diluted as equal amounts of the reagent and the distillate are mixed before the heating begins. Finally, when a distillation method is used, the product reacting with TBA does not have to be extracted with solvent, since the MA is obtained in a clear aqueous solution.

Tarladgis et al. (1962) investigated some of the conditions under which the structure of TBA may be altered because of hydrolytic or oxidative changes. They also ascertained the significance of these changes in the quantitative aspects of the test for the determination of MA in rancid foods. They presented evidence from ultra-violet, visible, and infra-red spectra as well as from paper and column chromatography of the various TBA reagents, that the structure of TBA is altered by acid-heat treatment. A more pronounced but similar effect

resulted from the treatment of the TBA with hydrogen peroxide. Some of the degradation products of TBA were found to absorb at the same wavelength as the TBA-MA complex, as do many of the compounds which are reported in the literature to react with TBA. In general, their results indicated that TBA should not be heated with acids or in the presence of oxidizing agents. Their results pointed out that more care should be taken in the running of the TBA test and in the treatment of the blank so that the results obtained are really quantitative for MA in rancid foods.

Tarladgis et al. (1964) showed that acid-heat treatment is not necessary for the condensation reaction of TBA with MA, or for maximum colour development. They also demonstrated that free MA is produced as a result of the oxidative breakdown of the unsaturated fatty acids of food products, and that acid-heat treatment is not required for the liberation of MA from a precursor. The amount of free MA produced can be measured without acid-heat treatment. The reaction between MA and TBA in water or 90% glacial acetic acid was investigated and the results showed that acid-heat treatment of the reaction mixture should be avoided, since the E_M^{530} of the coloured complex was considerably

affected by the acid. On the contrary, heating without acid accelerated the condensation of TBA with MA without affecting the E_M^{530}. As a result of their work they proposed a modification of the distillation procedure (Tarladgis et al., 1960), in which distillates of food products are reacted with TBA without the use of acid. Smith (1966) noted no significant difference between TBA values obtained with or without acid. Thus he concluded acid was not required for maximum colour development.

Kwon and Watts (1963) investigated the ultraviolet absorption of MA, as the basis of a new method for its quantitative assay. They reported that MA existed in aqueous solution mainly as the enolic form, β-hydroxy acrolein (CHOH = CHCHO), and that its ultraviolet absorption spectrum was pH-dependent. Below pH 3.0, the compound was believed to be s-cis-, planar, having an intermolecular H bond, with absorption maximum at 245 mμ and molar absorptivity (E) = 1.34×10^4. Above pH 7.0 the compound was completely dissociated and the maximum absorption of the enolate anion occurred at 267 mμ with E = 3.18×10 . They suggested that the absorbance difference between acidified and basified MA solutions at 267 mμ could be used as a measure of MA, even in the presence of other compounds that absorb in this spectral

region, provided their absorption is not pH-dependent. This difference is directly proportional to MA concentrations from 5×10^{-8}M to 3×10^{-5}M. They applied this method successfully to the assay of MA in distillates from rancid foods and although its sensitivity was only about 40% of the TBA test, it was sufficient to detect threshold levels of rancidity. They found the test to be simpler, much more rapid, and more specific than the TBA test. These findings on the configuration of MA at different pH's were substantiated in research done by Saunders and May (1963).

When distillation is employed to separate MA from other food constituents, maximum volatilization, even of free preformed MA, would not be expected at pH values above 3, since the volatile hydrogen-bonded ring form undergoes progressive ionization with increasing pH from 3 to 6.5 (Kwon, 1963), as quoted in Kwon and Watts, 1964). To test this assumption, Kwon and Watts (1964) distilled the same amounts of MA (3.6×10^{-6} moles/100 ml) at different pH's. Below pH 3 recovery of the compound was constant at about 65%, while above pH 6.5 the recovery was negligible. Between pH 3 and 6.5 the recovery depended on the proportions of the volatile chelated form and the nonvolatile enolate anion. They reported

that when extraction, rather than distillation, was used as the initial separation step, water seemed to be an adequate solvent for free preformed MA or its metal complexes. They proposed that the fact that higher yields of MA were obtained in fish by acid extraction or acid distillation was evidence that the acid treatment either produced more MA from a precursor, or that preformed MA was freed from a secondary combination with some other food constituents by the acid treatment. Kwon and Watts (1964) felt that the high correlations between MA content and rancid odours appeared to be limited to moist foods, especially animal tissues. They reported that in the pH range of such tissues, MA produced from lipid oxidation is mainly dissociated into the nonvolatile enolate anion, and might conceivably be stabilized against further irreversible reactions by the formation of metal chelates, from which MA can be recovered by acid and heat. In dehydrated foods the MA produced would be expected to be in the volatile chelated form, which would not be held in the food by metal chelation, thus explaining the low MA content of dehydrated foods.

Kwon *et al.* (1965) investigated the reactivity of MA with food constituents. They found that free MA is readily converted into its volatile form by acidification

only, whereas both acidification and heating are necessary to volatilize bound MA from its reaction products with proteins. Although Tarladgis et al. (1960) stated that maximum recovery of TBRS in the steam distillate from oxidized food was obtained only at acidic pH, they also reported preparing simple aqueous extracts from foods for the TBA test (Tarladgis et al., 1964).

Kwon et al. (1965) were of the opinion that such a procedure would be useful only in the initial stages of lipid oxidation when insoluble TBRS-protein products are unlikely to occur. From their experiments water extraction could not be considered satisfactory, since aqueous extracts contained only the free TBRS and water-soluble products, but did not include TBRS bound to water-insoluble proteins and other food constituents.

Kwon et al. (1965) suggested that in the pH range of moist foods, especially animal tissues, free TBRS produced from lipid oxidation would not volatilize and that probably hydration of the TBRS alone, as with the MA anion, could lead to the accumulation of the compound at low concentration. When the TBRS concentration in foods increased with advanced lipid oxidation, the anion could react further with amino acids, peptides, proteins, glycogen and other food constituents. If the

TBRS concentration was further increased, binding sites of the proteins may be saturated by the compound. Eventually, cross-linking with the other protein molecules could occur with resultant loss of solubility and recoverable TBRS. The ability of the compound to combine with proteins even at -18°C, as indicated by the *in situ* formation of TBRS-protein product in frozen tuna, accounts for the fate of some of the compound in the moist food systems.

Kwon *et al.* (1965) concluded that the absolute magnitude of the TBA number produced during oxidative rancidity of a foodstuff depended upon the composition of the lipid contained therein. For the proper use of this index of rancidity, one must take into account the reactivity of TBRS with the food constituents enumerated above, and the conditions necessary for its quantitative recovery. Indeed, the accumulation of the compound through its interaction with food constituents makes this test more desirable than others in assessing rancidity in foods.

The above discussion is predicated on the assumption that the reactions of TBRS are similar to those of MA. However, several lines of evidence suggest that the two are not the same. Saslaw and

Waravdekar (1965) presented evidence, from thin-layer chromatography studies of extracts of irradiated fatty acids, that none of the TBRS was MA. Nevertheless, MA and TBRS were alike in being water-soluble, dialyzable, TBA-reactive, able to react with proteins, and having a pH-dependent volatility. However, with respect to the final evaluation of TBRS as an index of rancidity in foods, the possibility that it may be liberated from an unknown precursor by acidic steam distillation, or that it may be formed by some hitherto unrecognized biochemical mechanism, unrelated to oxidation of unsaturated fats, must be taken into account (Kwon et al., 1965).

It has been strongly suggested by Kwon et al. (1965) that the prevailing concept that MA is the sole end product of lipid oxidation needs careful re-evaluation.

The TBA assay for lipid oxidation often involved the use of lipid solvents (Dahle et al., 1962; Tarladgis et al., 1960). In experiments using the TBA reaction, it was noted by Ho and Brown (1966) that a variety of solvents would, alone, react with TBA. They found that pigments with absorption maxima at 450 and 532 mμ were readily formed from petroleum ether and diethyl ether. Methyl alcohol, ethyl alcohol, chloroform, and hexane reacted, but to a lesser extent. The production of these

MA-like compounds, presumably from contaminants in the solvents, was increased by heating. They concluded that since TBA is a very reactive compound every precaution should be taken in the use of the TBA reaction where lipid solvents are involved. Thus for the quantitative determination of lipid oxidation by the TBA method, not only should acetic acid be purified but the lipid solvents also. They proposed doing this by refluxing the solvent with TBA to obtain purified distillates.

Most workers report values for the TBA reaction in arbitrary absorbance units which, in view of the diversity and empirical nature of the methods employed, cannot be compared from one laboratory to another. Sinnhuber and Yu (1958) proposed the use of 1,1,3,3-tetraethoxypropane (TEP) to prepare a standard curve for the TBA determination of MA. Acid hydrolysis of this acetal yields MA which reacts quantitatively with TBA and affords a procedure for the determination of this carbonyl compound. The results of TBA tests on various food products may be quantitatively measured by this method and the degree of oxidative rancidity expressed in milligrams of MA per 1000 g of sample. The use of TEP for a standard curve has been reported by several investigators (Taladgis et al., 1960; Zipser and Watts,

1961; Tarladgis et al., 1964; Kwon and Watts, 1963; Dahle et al., 1962; Smith, 1966).

Separation of 2,4-DNPHs by Thin-layer Chromatography

Among the reagents suitable for the identification and characterization of carbonyl compounds, 2,4-dinitrophenylhydrazine (2,4-DNPHine) has often been recommended and used (Allen, 1930; Brady, 1931; Strain, 1935; Campbell, 1936; Allen and Richmond, 1937; Braude and Jones, 1945; Roberts and Green, 1946; Stadtman, 1948; David, 1949; Neuberg et al., 1952; Fuleki, 1961; Maruta and Suzuki, 1961; Meijboom and Jurriens, 1965; Smith, 1966; Whitfield and Shipton, 1966).

Pool and Klose (1951) developed a quantitative procedure for the determination of monocarbonyl compounds in rancid foods, based on the formation of 2,4-dinitrophenylhydrazones (2,4-DNPHs) of the monocarbonyl compounds in a crude benzene extract of the rancid food. They found that the carbonyl content of rancid foods did not show any consistent correlation with peroxide contents, the ratio apparently being dependent on the stage of oxidative deterioration and the conditions under which deterioration took place. However, it was a simple accurate method for determining the gross aldehyde

content of rancid foods and was superior to the peroxide test for chemical determination of fat deterioration, since aldehydes contribute much more to off-odours and flavours present in rancid fat than do the relatively non-volatile peroxides.

In the chromatophoric separation of coloured compounds a number of methods have been explored. Earlier research workers used adsorption chromatography on columns, with a number of different substances as adsorbents: talc, alumina, aluminum phosphate, magnesium phosphate, and fuller's earth (Strain, 1935); alumina (Lucas et al., 1935; Johnston, 1947); silicic acid (Roberts and Green, 1946; David, 1949; Pippen et al., 1957; Fuleki, 1961); magnesium sulfate (Wahhab, 1948; Stadtman, 1948); Volelay bentonite (White, 1948, 1950); and magnesia-celite (Schwartz et al., 1962).

More recently, the thin-layer chromatography (TLC) of 2,4-DNPHs of aromatic carbonyl compounds has been reported by Dhont and DeRooy (1961) and DNPHs of simple aliphatic carbonyl compounds have been separated by Rosmos and Deyl (1961). 2,4-DNPHs of hydroxycarbonyl compounds have been separated by Anet (1962). Urbach (1963) reported the separation of mixtures of 2,4-DNPHs of the homologous series of n-alkan-2-ones, alk-1-en-3-ones,

alkanals, alk-2-enals, alka-2,4-dienols and alka-2,6-dienols on kieselguhr impregnated with phenoxyethanol, and aluminum oxide impregnated with silver nitrate. Denti and Luboz (1965) described a method for the separation of DNPHs of aldehydes and ketones by TLC. Byrne (1965) described the TLC technique for the separation of a 41 mono-, bis-, and tris-2,4-DNPHs derived from simple saturated aldehydes and ketones, unsaturated aldehydes, di-, bis-, and hydroxycarbonyl compounds, and α-ketoacids. Meijboom and Jurriens (1965) used TLC for the separation of the positional and geometrical isomers of monoenic aldehydes via the 2,4-DNPHs. Schwartz and Parks (1963) separated the aliphatic carbonyl 2,4-DNPHs into classes by TLC.

Whitfield and Shipton (1966) found the volatiles from stored unbleached frozen peas to contain 12 carbonyl compounds: ethanal, propanal, hexenal, pent-2-enal, hex-2-enal, hept-2-enal, oct-2-enal, non-2-enal, hept-2,4-dienal, non-2,4-dienal, dec-2,4-dienal, and propan-2-one. The identifications were based on the thin-layer partition chromatography and infrared, ultraviolet, and visible spectra of the 2,4-DNPHs. These carbonyl compounds were thought to be involved in the formation of off-flavour in frozen raw peas.

In TLC, the adsorbents employed, their preparation, the thickness of the adsorbent on the plates, the activation of the adsorbent, the solvent system(s) used, and the method of preparation of the 2,4-DNPHine derivatives vary from one worker to another.

Anet (1962) used aluminum oxide G (Merck) and silica gel G (Merck) mixed with water in a ratio of 1 g of adsorbent to 2 g of water as adsorbents. These were applied as slurries, 1.5 min after mixing, with an applicator giving a film of 0.25 mm thickness on glass plates. Some of the plates were air dried and, in some cases, were activated further by drying at 100°C for 2 h. For some separations the plates were deactivated in an atmosphere of 70% relative humidity at 20°C for a few hours. To obtain reproducible R_f values, he indicated that the water content of the adsorbents must be constant, since water lowers their activity. Anet (1962) found that mixtures of toluene and ethyl acetate were the most useful solvent systems. Increasing the proportion of ethyl acetate resulted in increases in eluting power. The mixture used depended on the compounds to be separated, the adsorbent, and its degree of activation. Both one-dimensional and two-dimensional separations were carried out. The developed chromatograms

were sprayed with a 2% solution of NaOH in 90% ethanol, to give intense characteristic colours, the blue or purple colours being apparently specific for the 1,2-bis-hydrazones. Most of the hydrazones were prepared by the general procedure of Neuberg et al. (1952), and dissolved in acetone for application. The mono- and bis-2,4-DNPHs derived from hydroxycarbonyl compounds were chromatographed by Anet (1962).

Denti and Luboz (1965) used layers of silica gel G (Merck), alumina G (Merck), silica gel G (Merck) + 25% $AgNO_3$, and alumina G (Merck) + 25% $AgNO_3$ at a thickness of 0.3 mm on glass plates. The plates coated with silica gel G and silica gel G + 25% $AgNO_3$ were air-dried for 5 min, then oven-dried for 30 min at 110°C and stored in a desiccator over $CaCl_2$ before use. The plates with layers containing $AgNO_3$ had to be kept in the dark. The plates coated with alumina G and alumina G + 25% $AgNO_3$ were air-dried for 5 min, then oven-dried for 30 min at 110°C and subsequently deactivated for 12 hours by exposing them to air at room temperature and approximately 60% relative humidity. The plates carrying a layer containing $AgNO_3$ were kept in the dark. Several solvent systems were used with the most useful being benzene-petroleum ether, chloroform-petroleum

ether, benzene-hexane, and cyclohexane-nitrobenzene-petroleum ether. The plates were developed one-dimensionally at a temperature of 18°-22°C and, following development, were sprayed with a 2% solution of NaOH in 90% ethanol, to obtain more easily seen spots (yellow to a more or less dark brown). Special care was taken to standardize the techniques for layer deposition and activation of plates in order to obtain reproducible Rf values. The 2,4-DNPHs were prepared by the method of Brady (1931) and recrystallized twice from methanol. All the data reported by Denti and Luboz were internally standardized R_f (formaldehyde) = 1 and they found low concentrations of compounds were best detected on $AgNO_3$ treated adsorbents.

Byrne (1965) reported the use of silica gel G and aluminum oxide G (Merck) as adsorbents mixed with water in a ratio of 1 g adsorbent to 2 g of water. These were applied in a layer 0.25 mm thick on glass plates by means of a Desaga spreader. The plates were air dried for 30 min at room temperature and then activated by heating at 110°C for 2 h, or dried without heating. The solvent systems found suitable for the separation of all the DNPHs examined were: 80-100°C light petroleum-diethyl ether, benzene-tetrahydrofuran, and benzene-

tetrahydrofuran-glacial acetic acid. The plates were developed by normal, multiple, two-dimensional and continuous horizontal development and, following development, were sprayed with ethanolamine to obtain characteristic colours for different classes of 2,4-DNPHs. Most of the hydrazones were prepared from the parent carbonyl compounds by the method of Neuberg et al. (1952). The hydrazones were dissolved in benzene, or dissolved first in ethyl acetate or tetrahydrofuran followed by addition of benzene for application on the plates.

Meijboom and Jurriens (1965) used silica gel impregnated with 30% $AgNO_3$, at a thickness of 0.5 mm on glass plates, for their work on the separation of positional and geometrical isomers of monoenic aldehydes via the 2,4-DNPHs and TLC.

The importance of having a small starting spot in TLC in order to obtain good resoltuion of the compounds involved was pointed out by Byrne (1965). The relative humidity in the laboratory and/or in the developing chamber has been reported to be significant by a number of investigators. Anet (1962) found a loss of activity of plates took place during handling in hot, humid weather. Geiss and Schlitt (1960), working with poly-phenols found the results depended markedly on relative

humidity and that most of the difficulties caused by relative humidity could be overcome by introducing the coated plates into the developing chambers after they had been heated to 90°C. That relative humidity played an important part in the separation of hydrazones was also reported by Badings (1961).

II. MATERIALS AND METHODS

Preparation of Material

The beans used in this investigation were obtained from three different sources. Those in Series I were obtained from Green Giant Co. Ltd., Ste. Martine, Quebec, while those used in Series II and III were obtained from the Berlet Fruit Co., Montreal, Quebec. Beans from the A & P Food Store, Ste. Anne de Bellevue, Quebec, were used in the work on the identification of the 2,4-DNPHs.

In Series I the beans were harvested and transported on September 7, 1965 from Green Giant Co. to Macdonald College where they were stored overnight at 1.1°C. In this series, the beans had been previously snipped, while in the other two series whole beans were used. In Series II and III, the beans were processed immediately rather than being stored overnight.

The beans were handled as follows in the three series. They were divided into two lots. One lot was left raw and the other was blanched for 3 min in a cabinet-type steam blancher. This was followed by spray cooling with tap water, after which the material was spread out on paper and allowed to drain for 30 min.

The beans were then placed in 20-oz sanitary cans using 125 g of material per can. After closing the containers the covers were perforated with a single hole to permit evacuation and replacement of the atmosphere, to study the effect of composition of the atmosphere.

The cans were placed in a vacuum desiccator and evacuated, using a Cenco Pressovac 4 vacuum pump, to a pressure of about 0.5 cm in one min and maintained at this level for another 4 min. The vacuum was released slowly with oxygen, nitrogen, or air to provide different atmospheres. When oxygen or nitrogen was used, a slight positive pressure was allowed to build up in the desiccator before lifting the lid, to prevent air getting into the cans. The hole in each can was immediately sealed with solder.

In Series I, the containers were taken to the freezer after each hour of operation and placed on plates at -25°C and held for 24 h after which they were subdivided into 3 temperature groups and stored at -9.4°, -15.0°, and -20.6°C.

In Series II and III, the containers were taken to the freezer after each hour of operation and held at -20.6°C for 24 h after which they were subdivided into

3 temperature groups and stored at -9.4°, -15.0°, and -20.6°C.

Initial determinations for TBRS were carried out on the same day as the material was processed and subsequent determinations were carried out at regular intervals throughout the storage period.

Two other lots of material were handled similarly for use in identification of the 2,4-DNPHs.

Organoleptic Tests

Organoleptic tests were conducted on all the samples throughout the storage period on the same day the TBA determinations were carried out. Flavour scores were assigned as follows: "A" good, "B" slightly off, "C" off, "D" very off. These tests were carried out on both the frozen and the thawed material.

Extraction of TBA Reactive Substance

The method of Smith (1966) was used with some modifications for the extraction of TBRS from beans. The first series ran for 76 days. The second and third series were run concurrently using the same blanched material. These series were run for a 42-day period.

The procedure used was as follows: 100 g of material were blended with 100 ml of 95% ethanol in a Waring blendor at high speed for 2 min. The slurry was filtered through Whatman filter paper Number 3 in a Buchner funnel, using a vacuum of 3 pounds. The blendor was washed twice with 25 ml of 95% ethanol. These washings were added successively to the Buchner funnel to rinse the residue. When filtration was complete, the filtrate was transferred to a 250 ml volumetric flask and made up to volume using washings of 95% ethanol from the suction flask.

The filtrates were stored for 18 h at 5°C. They were removed from the storage and shaken. An aliquot was taken and centrifuged to remove any precipitate. No dilutions of the filtrate were made.

The TBA test was carried out using 5 ml of TBA reagent and 5 ml of bean extract. The samples were prepared in duplicate. A reagent blank was prepared using 5 ml of TBA reagent plus 3 ml of 95% ethanol and 2 ml of distilled water. The reagent blank was used to adjust the instrument to 100% T. The sample blanks contained 5 ml of bean extract and 5 ml of reagent, which did not contain any TBA reagent. The sample blank was used to eliminate any natural colour in the system or

colour production during heating which might absorb at 520 mμ, but which was not due to the TBA reaction. The TBA reagent used was made up as follows: 0.67 gm of TBA was dissolved in 140 ml of distilled water and 60 ml of glacial acetic acid. The flask was held in hot water to facilitate solution. In the sample blanks the reagent used was 70% distilled water and 30% glacial acetic acid.

Absorption readings were taken using a Coleman Junior Spectrophotometer Model 6A. Maximum absorption was found to be occurring at 520 mμ on this instrument. However, several other workers have all reported absorption maxima occurring at 530-538 mμ for a number of products (Patton and Kurtz (1951) 532 mμ for oxidized milk fat; Wilbur et al. (1949) 532 mμ for oxidized methyl linolenate; Sidwell et al. (1955) 530 mμ for oxidized milk powder; Turner et al. (1954) 535-538 mμ for rancid pork; Romero and Gonzalez-Quijano (1956) 530 mμ for rancid lard; Sinnhuber and Yu (1958) 532-535 mμ for rancid salmon oil; Schmidt (1959) 532 mμ for oxidized lard; Khomutov and Garkusha (1960) 531-535 mμ for oxidized fats). Thus an oxidized oil sample was used to check the readings obtained on the Coleman Junior Spectrophotometer. This sample also showed a maximum absorption at 520 mμ. The same material was

then tested on a Bausch and Lomb Spectronic 505 and a Beckman Model DU. Both instruments showed an absorption peak for the sample between 530 and 535 mμ. Since the grating on the Coleman Junior Spectrophotometer is not as fine as that of the Bausch and Lomb Spectronic 505 and the Beckman Model DU, this difference could be attributed to the difference in gratings. The absorption curve obtained on the Spectronic 505 showed a shoulder at 500 mμ, exhibited by the normal curve in this vicinity. This could move the absorption maxima of the Coleman Junior Spectrophotometer to the right.

A heating time of 35 min was chosen. This was based on a comparison carried out between fresh material and unblanched material stored at 17.8°C under oxygen for 3 years, to see if the differences between the absorption values of the samples became constant after heating for 30, 35, 40, and 45 min. From these results a heating time of 35 min was chosen. A 35-min heating time was also used by Tarladgis et al. (1960).

The samples were heated in a glycerine-water bath. The temperature of the bath was set at 100°C. An electric stirrer was used to maintain the temperature of the bath constant at all points.

The reactivity of the lipid solvent used, 95%

ethanol, with TBA was tested according to the method of Ho and Brown (1966).

Most of the results reported in the literature are reported as TBA numbers. The TBA number is defined as the number of mg of MA per 1000 g of material. Since MA could not be detected in the bean samples, this definition does not apply. In this investigation, the TBA number might better be described as the number of mg of TBA reactive substance (TBRS) per 1000 g of material calculated as MA.

A standard curve was prepared as proposed by Sinnhuber and Yu (1958), using known amounts of 1,1,3,3-TEP. On hydrolysis, one mole of 1,1,3,3-TEP produces one mole of MA.

Preparation and Separation of 2,4-DNPHs

As indicated in the review of literature, one of the most suitable reagents for the identification and characterization of carbonyl compounds is 2,4-dinitrophenylhydrazine (2,4-DNPHine). Thin-layer chromatography has been used for the separation of the 2,4-DNPHs of carbonyl compounds by Anet (1962), Denti and Luboz (1964), Byrne (1965), and Meijboom and Jurriens (1965).

The use of 2,4-DNPHine has many advantages. Its derivatives are very insoluble in water and can, therefore, be isolated from dilute solutions. They are easily crystallized from alcohol. The relatively high molecular weight of 2,4-DNPHine makes it possible to obtain a reasonable amount of the derivative from small quantities of carbonyl compounds.

The 2,4-DNPHine was made up according to the method of Neuberg et al. (1952) using 1.2 g of 2,4-DNPHine dissolved in 50 ml of 30% perchloric acid ($HClO_4$) solution.

The hydrazones examined in this investigation were prepared mainly from the ethanol extracts of the beans. The bean extract was filtered through Whatman filter paper Number 5 under a 5-pound vacuum, to remove any precipitate present. One hundred and fifty ml of the extract plus 50 ml of the 2,4-DNPHine solution were placed in a round-bottom flask and refluxed for 30 min, using a Glas-Col heating unit. After cooling, the material was placed at 5°C for 18 hours. The crystals formed were filtered off, under high vacuum, using Whatman filter paper Number 50, and washed twice using a $HClO_4$ solution (30 ml of 60% $HClO_4$ in 220 ml of water). The crystals were then dissolved in 50 ml of acetone.

In some cases the 2,4-DNPHs were made from steam distillates of the beans. Two hundred and fifty g of beans were blended with 300 ml of water in a Waring blendor at high speed for 3 min. The slurry was transferred to a distilling flask, using 100 ml of water. Dow Corning Anti-Foam A was used to prevent any foaming.

The distillate was collected in a 500 ml flask containing 25 ml of water, the tip of the condenser dipping in the water. The receiver was kept in an ice bath to minimize losses by volatilization. A total of 250 ml of distillate was collected in 30 min. Twenty-five ml of the distillate were stored at 5°C for 18 hours, for the determination of TBRS. The 225 ml of distillate plus 50 ml of 2,4-DNPHine solution were placed in a round bottom flask and refluxed for 30 min using a Glas-Col heating unit. After cooling, the material was placed at 5°C for 18 hours. The 2,4-DNPHs were recovered by the method previously outlined.

The plates for thin-layer chromatography were prepared according to the method used by Byrne (1965). Thirty g of silica gel G (E. Merck, A. G. Darmstadt) were mixed with 60 ml of water. After shaking vigorously for 3 min, the slurry was applied at a thickness of 0.25 mm using a Shandon spreader. The plates were air dried

for 30 min, followed by oven drying at 110°C for 70 min, and stored over anhydrous $CaSO_4$ until used.

The amount of material spotted varied in each series from a minimum of 5 λ per spot to a maximum of 15 λ per spot. When the plates were to be photographed, a single spot was employed and the chromatograms were developed two-dimensionally using two solvent systems. For identification and comparison work, a number of spots were placed 1.5 cm apart and one-dimensional chromatography using one solvent system was carried out. The solvents were allowed to travel the full length of the plates in both cases.

The chromatograms were developed in Desaga developing tanks, using the ascending technique. In order to achieve saturation conditions in the developing chamber, it was necessary to line three sides of the chamber with Whatman filter paper Number 1. The solvent system was placed in the tanks at least one hour before developing the chromatograms. The tank covers were held tightly in place by several 2-pound lead weights. The temperature of the chromatography room was kept at 20°C.

Several solvent systems were investigated. Chloroform-ethanol (5:1)(V/V), (10:1)(V/V), (15:1)(V/V), chloroform-methanol (10:1)(V/V), (15:1)(V/V), and

toluene-ethyl acetate (1:1)(V/V) resulted in very fast movement, but the separation was incomplete. Chloroform-ethanol (20:1)(V/V) and toluene-ethyl acetate (2:1)(V/V) gave a fairly good separation but still too rapid. With chloroform-ethanol (25:1)(V/V) and toluene-ethyl acetate (4:1)(V/V) the movement was a little too slow for good separation.

The solvent system found to give the best development of the 2,4-DNPHs from the ethanol extract was chloroform-methanol (22:1)(V/V) and toluene-ethyl acetate (3:1)(V/V). The chromatograms were first developed in chloroform-methanol. After thorough drying the plates were turned 90° and developed in the toluene-ethyl acetate.

The chromatograms of the 2,4-DNPHs of the steam distillates were developed using the same solvent systems and the same methods.

Chromatograms of the 2,4-DNPHs from 95% ethanol and 2,4-DNPHine, without bean extract, were developed in the same solvent system. This was done to determine what spots if any, on the chromatograms, were due to these compounds.

Identification of 2,4-DNPHs

An attempt was made to identify some of the 2,4-DNPHs of the ethanol extract of beans, in Series I. The 2,4-DNPHine derivatives were made from ethanol extracts, both blanched and unblanched, stored under the three atmospheres at -9.4°C. These samples were used because it was assumed that they would contain the largest amounts of carbonyl compounds.

Twelve spots of 10 λ each, were plated 1.5 cm apart and at a distance of 2 cm from the edge of the plate. The chromatograms were developed one-dimensionally in chloroform-methanol (22:1)(V/V) and the solvent travelled the full length of the plate. After drying, the chromatogram was divided into six well-defined areas. The material from each area was then collected and eluted with acetone. Nine plates were developed and treated in this manner, for each sample.

The six elutions were in turn spotted and developed one-dimensionally in chloroform-methanol, to permit collection of the individual chromatogen spots in a pure state. These spots were then eluted with acetone, dried and dissolved in 95% ethanol.

The absorption spectrum of each of these solutions was determined using the Bausch and Lomb Spectronic 505.

The absorption spectra obtained were compared with those found in the literature for known carbonyl compounds (Braude and Jones, 1945; Roberts and Green, 1946) in an effort to characterize them.

An attempt was also made to identify the 2,4-DNPHs from beans by using the 2,4-DNPHs derived from the steam distillate of beans stored under oxygen at -9.4°C for a long period of time, and comparing them with the 2,4-DNPHs of known carbonyl compounds. The 2,4-DNPHine derivatives of acetaldehyde, diacetyl, glyoxal, valeraldehyde, anisaldehyde, benzaldehyde, butyraldehyde, n-hexaldehyde, formaldehyde, hexanal, 1-heptanal, and propionaldehyde, were prepared by adding each compound to a small amount of 2,4-DNPHine solution. After allowing 24 hours for the formation of the 2,4-DNPHs, the crystals were filtered off, washed with water and recrystallized from hot ethanol and water. They were dissolved in acetone for spotting on the thin-layer plates.

The 2,4-DNPHs of the steam distillates were obtained as follows. Two hundred g of beans were put through a meat grinder, while in a frozen state, and transferred to a distillation flask, using 125 ml of water. Four hundred ml of distillate were collected in a 500-ml round-bottom flask. The receiver flask, held

in an ice bath, contained 50 ml of water, the tip of the condenser dipping in the water. These measures were taken to minimize losses by volatilization.

The steam distillate obtained was redistilled in an all-glass apparatus, using a fractionating column. The distillate was collected into a receiving flask containing 50 ml of a saturated 2,4-DNPHine solution in $HClO_4$, the tip of the condenser dipping into the solution to prevent losses by volatilization. The distillation was stopped after about 100 ml had distilled over. The solution was stored for 24 hours to allow the formation of the 2,4-DNPHs. The 2,4-DNPHs were filtered off, washed with warm water and dissolved in 25 ml of acetone. This technique is similar to that used by David (1949), Fuleki (1961), and Whitfield and Shipton (1966).

The 2,4-DNPHine solution used in this preparation contained 1.6 g of 2,4-DNPHine per 50 ml of 30% $HClO_4$. This is in accordance with the suggestion of Neuberg et al. (1952) to use the reagent in higher concentration if it is to be employed for the precipitation of the hydrazine derivative from dilute solutions.

The plates were spotted at a distance of 2 cm from the edge using 15 λ of the 2,4-DNPH solutions of known and unknown compounds. The spots were spaced

1.5 cm apart. The chromatograms were developed in toluene-ethyl acetate (3:1)(V/V) one-dimensionally.

Recovery of Volatiles for Ultraviolet Absorption Studies

An attempt was made to obtain the volatiles of beans using a different steam distillation procedure, in order to study their characteristics. A 100 g sample of beans, stored at -9.4°C under oxygen for 10 months, was blended with 150 ml of water at high speed in a Waring blender for 3 min. The slurry was transferred to a distilling flask using 50 ml of water and acidified with 10 ml of 3 N HCl to obtain a pH of 1.5. The acid pH was used since Tarladgis et al. (1960) found that maximum recovery of TBRS from oxidized food by steam distillation was obtained only under acid conditions. The use of this pH was also advocated by Kwon and Watts (1964). Kwon et al. (1965) stated that free MA is readily converted into its volatile form by acidification only, whereas both acidification and heating are necessary to free the protein-bound MA.

Dow Corning Anti-foam A was used to prevent any foaming. The distillate was collected in a 100 ml graduated cylinder, the tip of the condenser dipping

into 25 ml of water. The receiver was kept in an ice bath to minimize volatilization.

Fifty ml of distillate were collected in 10 min. The distillate was adjusted to pH 1.5 using 3 N HCl since Kwon and Watts (1963) reported that at pHs lower than 2.5 MA exhibited maximum absorption at 245 mμ. The absorption curve was determined from 220 mμ to 520 mμ on the Bausch and Lomb Spectronic 505. A water blank adjusted to pH 1.5 with 3 N HCl was used.

Comparison of Steam Distillation and Ethanol Extraction

The two methods of extraction were employed over a period of time in order to compare the results and to see if any correlation were present.

The beans used in this series were obtained locally. They were divided into two lots. One lot was blanched and the other left unblanched. The material was placed in cans and an oxygen atmosphere was introduced by the method previously outlined. The material was held overnight at -20.6°C, following which it was transferred to the -9.4°C storage temperature. Determinations were carried out initially, and after 13 and 20 days. These intervals were chosen since they

allow sufficient time for off-flavour development.

The ethanol extraction method was the same as that previously outlined. Steam distillation was carried out as previously outlined without acidification. In both methods the final volume was 250 ml. An aliquot of 25 ml was used for the TBA test and the remainder used to prepare the 2,4-DNPHine derivatives, using the procedures described previously.

After obtaining the results of the TBA test, the TBA solutions were run through the Bausch and Lomb Spectronic 505, in order to obtain the absorption curves of the solutions over the period of off-flavour development. Absorption curves of both steam distillates and ethanol extracts were obtained.

The 2,4-DNPHs of the steam distillates and ethanol extracts were spotted on thin-layer plates. The plates were developed two-dimensionally using a single spot per plate at a distance of 2 cm from the edge. The ascending technique was employed. The first solvent system was chloroform-methanol (22:1)(V/V), and the second, toluene-ethyl acetate (3:1)(V/V).

Photographs of the developed plates were taken. A Polaroid Land MP-3 camera was used at first. The plates were photographed before and after spraying with

a 2% solution of NaOH in 90% ethanol as suggested by Anet (1962). However, the pictures obtained were considered unsatisfactory on the whole.

The work was repeated using plates developed as described but omitting the alkaline spray. Satisfactory photographs were obtained using an Ikoflex camera and PX professional 120 black and white film, with photo-flood lighting and an exposure of 1/50 at F11. Colour photographs were taken with Kodacolour X film with an exposure of 1/100 at F11.

III. EXPERIMENTAL RESULTS

2-Thiobarbituric Acid Test

A heating time of 35 min in a glycerine-water bath at 100°C was decided upon after carrying out a comparison of the colour development in alcohol extracts obtained from fresh beans, and alcohol extracts from unblanched beans stored at -17.8°C in oxygen for 3 years. The results are reported in Table 1. This 35-min heating time was found to give good colour development and was therefore employed throughout. Tarladgis et al. (1960) also proposed the use of a 35-min heating time in a boiling water bath to develop the colour of the TBA complex.

A standard curve was prepared from 1,1,3,3-TEP according to the method proposed by Sinnhuber and Yu (1958) and is shown in Figure 1. The mg of TBRS were estimated from this curve using the 2-log G values for the samples.

Glacial acetic acid was used throughout in the preparation of the TBA reagent at 30% concentration. Although Tarladgis et al. (1962, 1964) criticized the

TABLE 1

DETERMINATION OF HEATING TIME FOR THE TBA REACTION

Heating time in minutes	(2-log G) Sample stored in oxygen for 3 years	(2-log G) Fresh material	Difference (2-log G)
30	.319	.194	.125
35	.345	.198	.147
40	.385	.240	.145
45	.423	.305	.117

use of acid in the preparation of TBA reagent, the writer felt that better colour development was obtained with the use of acid. Also since an acid blank was employed the side effects of the acid would be cancelled out.

TBA Experiments on Stored Beans

In Series I the TBA test was used for the analysis of blanched and unblanched beans stored under oxygen, nitrogen and air at -9.4°, -15.0°, and -20.6°C. The results are reported in Tables 2, 3, 4, 5, 6 and 7 and plotted in Figures 2, 3, 4, 5, 6 and 7. The values obtained at the 3-, 6-, and 9-day intervals in Series I were excessively high. This is likely due to improper

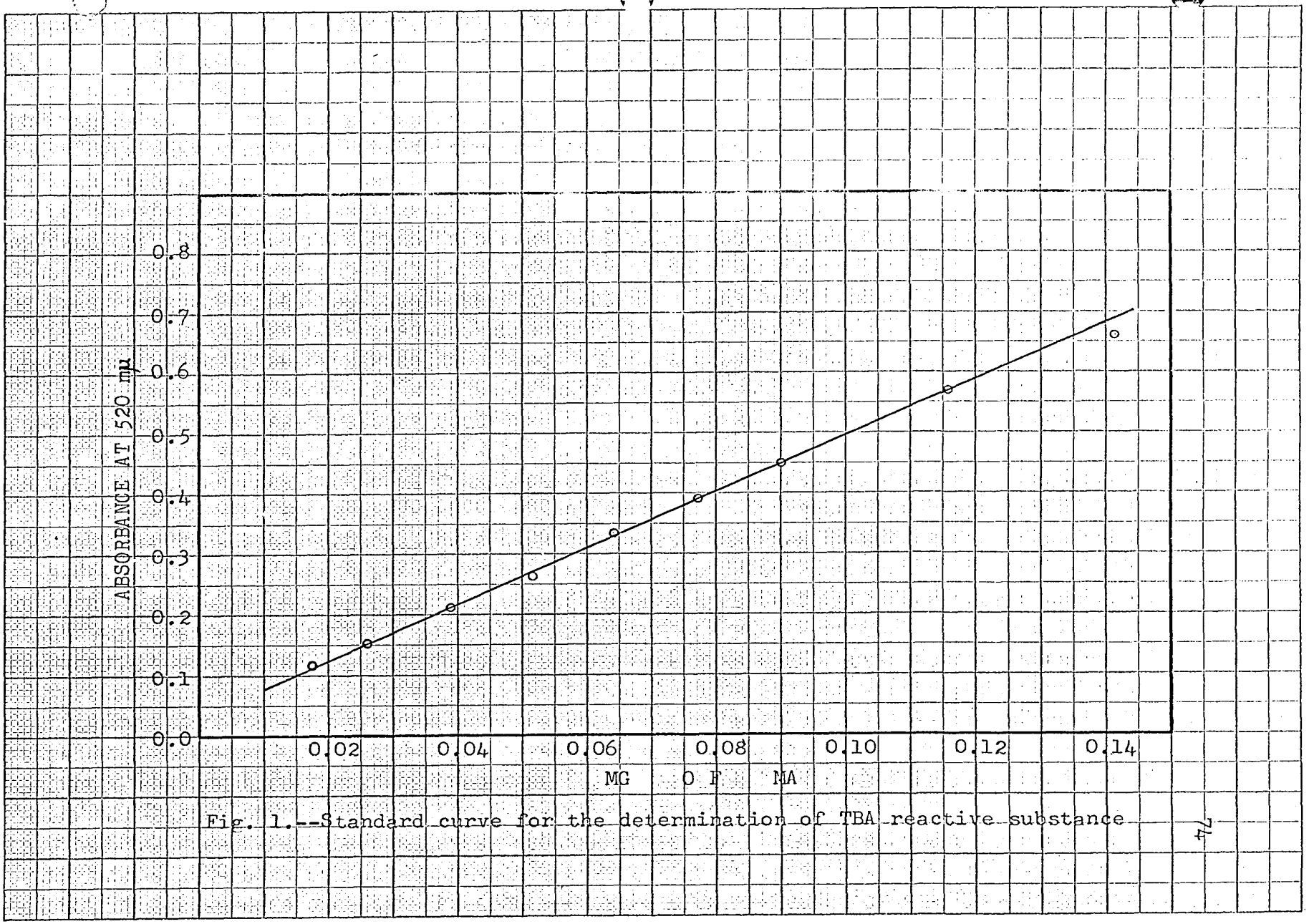

Fig. 1.--Standard curve for the determination of TBA reactive substance

TABLE 2

EFFECT OF STORAGE TEMPERATURE ON TBA NUMBERS AND FLAVOUR OF UNBLANCHED BEANS STORED UNDER OXYGEN, SERIES I

Storage temperature °C	Storage time in days												
	Initial	3	6	9	12	15	18	21	27	34	41	48	76
- 9.4	1.00A	2.18A	1.55A	1.64A	1.47A	1.52B	1.54C	1.53C	1.53D	1.51D	1.51D	1.56D	1.57D
-15.0	1.00A	2.69A	1.72A	1.48A	1.46A	1.47B	1.53C	1.53C	1.56D	1.55D	1.58D	1.53D	1.56D
-20.6	1.00A	3.18A	1.74A	1.32A	1.03A	1.26B	1.24B	1.26B	1.22C	1.35D	1.34D	1.36D	1.42D

Flavour scores: A = good; B = slightly off; C = off; D = very off

TABLE 3

EFFECT OF STORAGE TEMPERATURE ON TBA NUMBERS AND FLAVOUR OF BLANCHED BEANS STORED UNDER OXYGEN, SERIES I

Storage temperature °C	Storage time in days								
	Initial	6	12	18	27	34	41	48	76
- 9.4	0.78A	0.86A	0.71A	0.78A	0.77A	0.73A	0.73A	0.73A	0.68A
-15.0	0.78A	0.75A	0.71A	0.67A	0.70A	0.76A	0.75A	0.71A	0.69A
-20.6	0.78A	0.93A	0.67A	0.71A	0.78A	0.75A	0.78A	0.74A	0.73A

Flavour scores: A = good; B = slightly off; C = off; D = very off

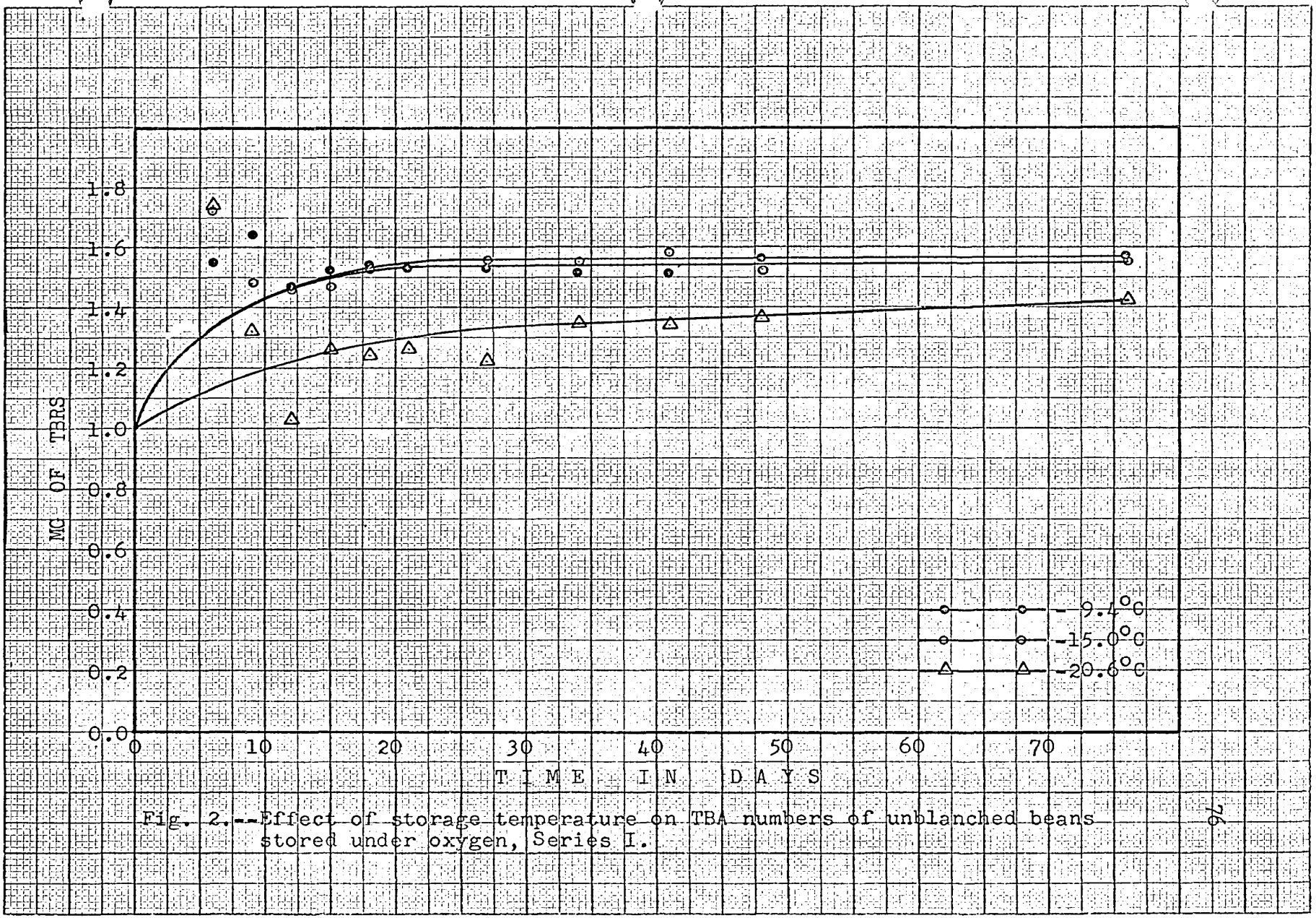

Fig. 2.--Effect of storage temperature on TBA numbers of unblanched beans stored under oxygen, Series I.

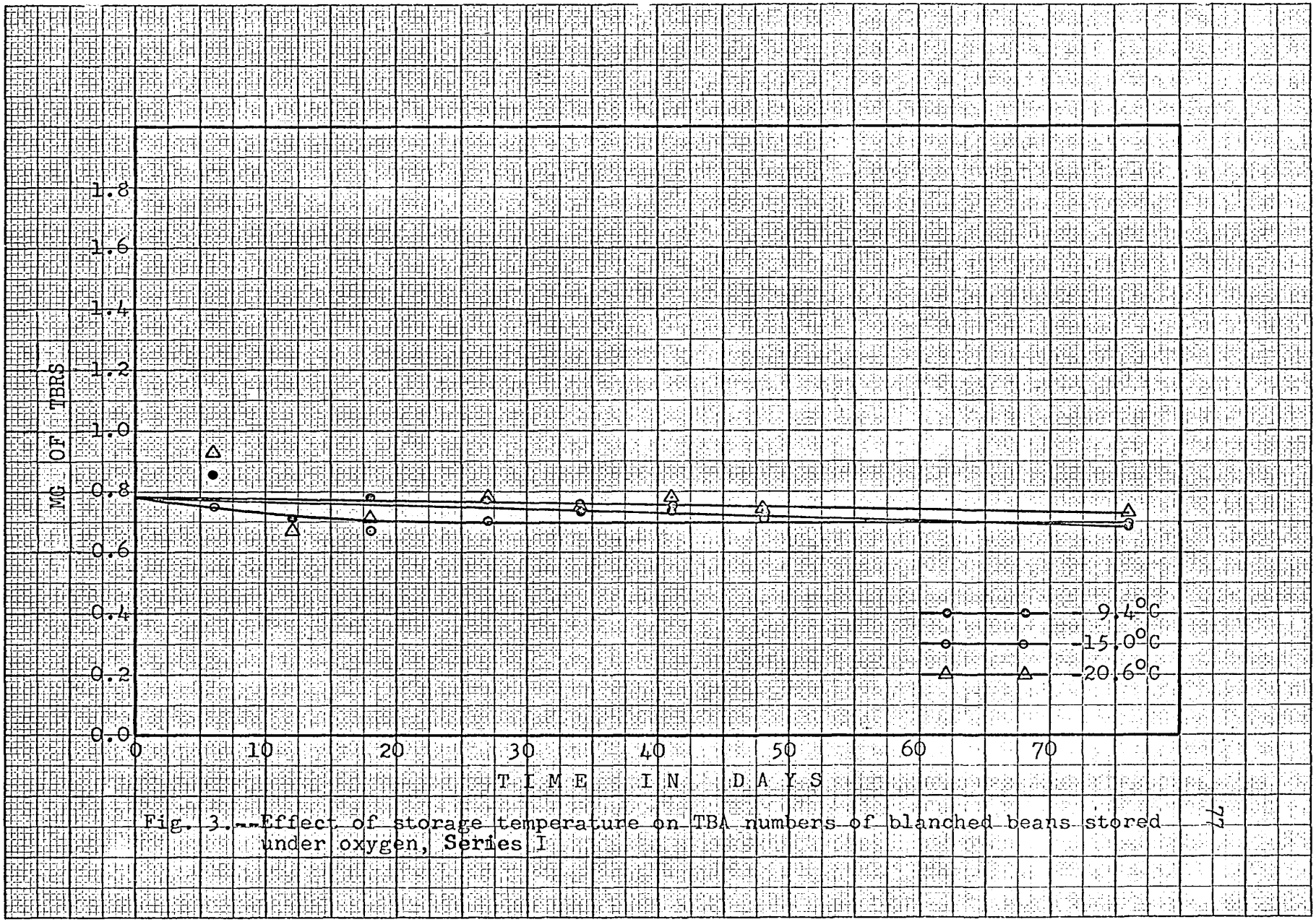

Fig. 3.--Effect of storage temperature on TBA numbers of blanched beans stored under oxygen, Series I

TABLE 4

EFFECT OF STORAGE TEMPERATURE ON TBA NUMBERS AND FLAVOUR OF UNBLANCHED BEANS STORED UNDER NITROGEN, SERIES I

Storage temperature °C	Storage time in days												
	Initial	3	6	9	12	15	18	21	27	34	41	48	76
- 9.4	1.00A	1.83A	1.32A	1.71A	0.93A	1.04A	1.37B	1.38B	1.37B	1.37B	1.38C	1.40D	1.40D
-15.0	1.00A	2.88A	1.48A	0.96A	0.92A	1.10A	1.26B	1.21B	1.26B	1.24C	1.22C	1.22C	1.21C
-20.6	1.00A	1.69A	0.89A	0.87A	0.71A	0.90A	1.04A	1.04A	1.04A	1.05B	1.04B	1.07B	1.04B

Flavour scores: A = good; B = slightly off; C = off; D = very off

TABLE 5

EFFECT OF STORAGE TEMPERATURE ON TBA NUMBERS AND FLAVOUR OF BLANCHED BEANS STORED UNDER NITROGEN, SERIES I

Storage temperature °C	Storage time in days								
	Initial	6	12	18	27	34	41	48	76
- 9.4	0.78A	0.96A	0.71A	0.74A	0.69A	0.79A	0.72A	0.68A	0.75A
-15.0	0.78A	0.92A	0.74A	0.71A	0.69A	0.72A	0.78A	0.75A	0.72A
-20.6	0.78A	0.79A	0.68A	0.65A	0.73A	0.69A	0.73A	0.67A	0.66A

Flavour scores: A = good; B = slightly off; C = off; D = very off

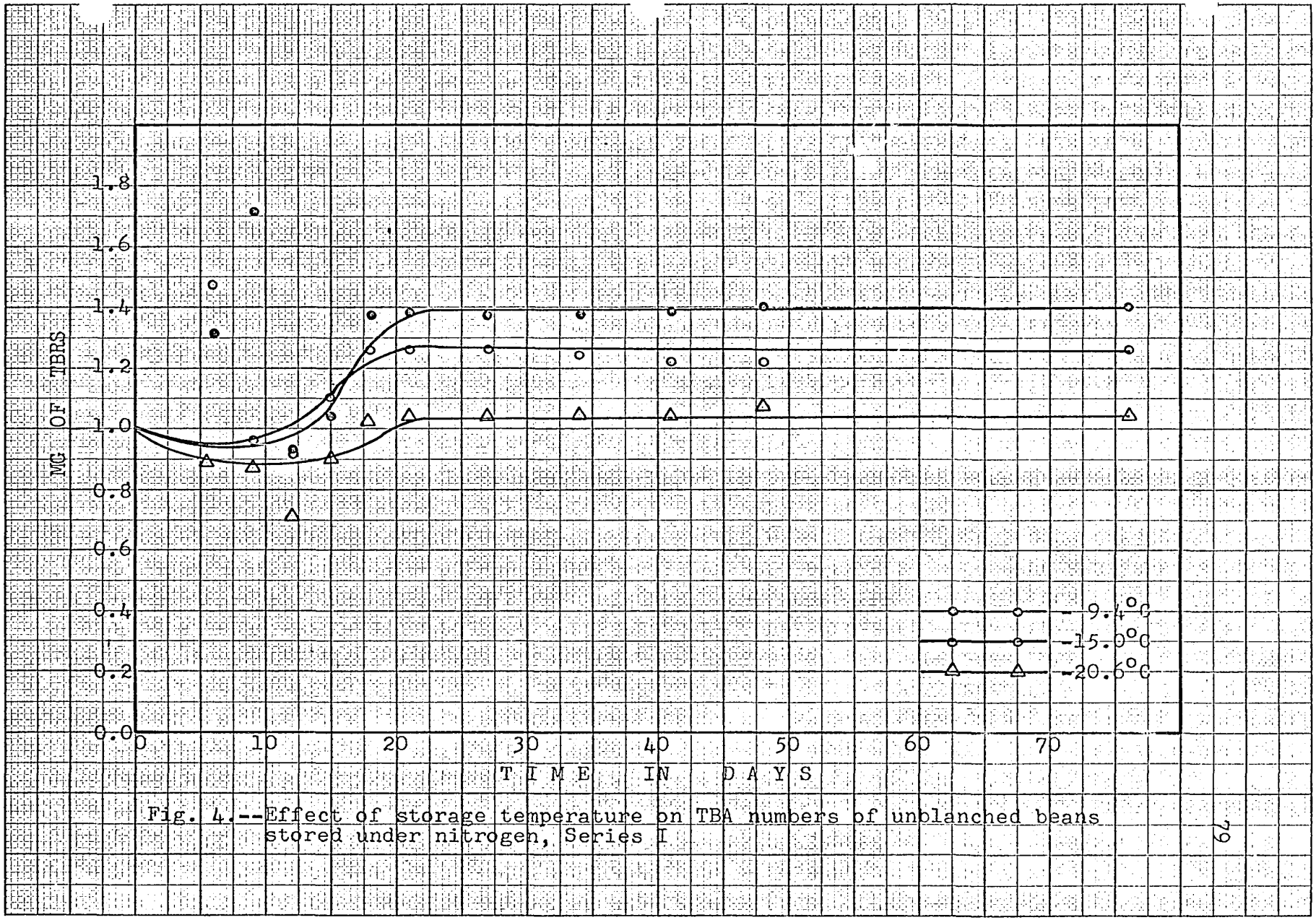

Fig. 4.--Effect of storage temperature on TBA numbers of unblanched beans stored under nitrogen, Series I

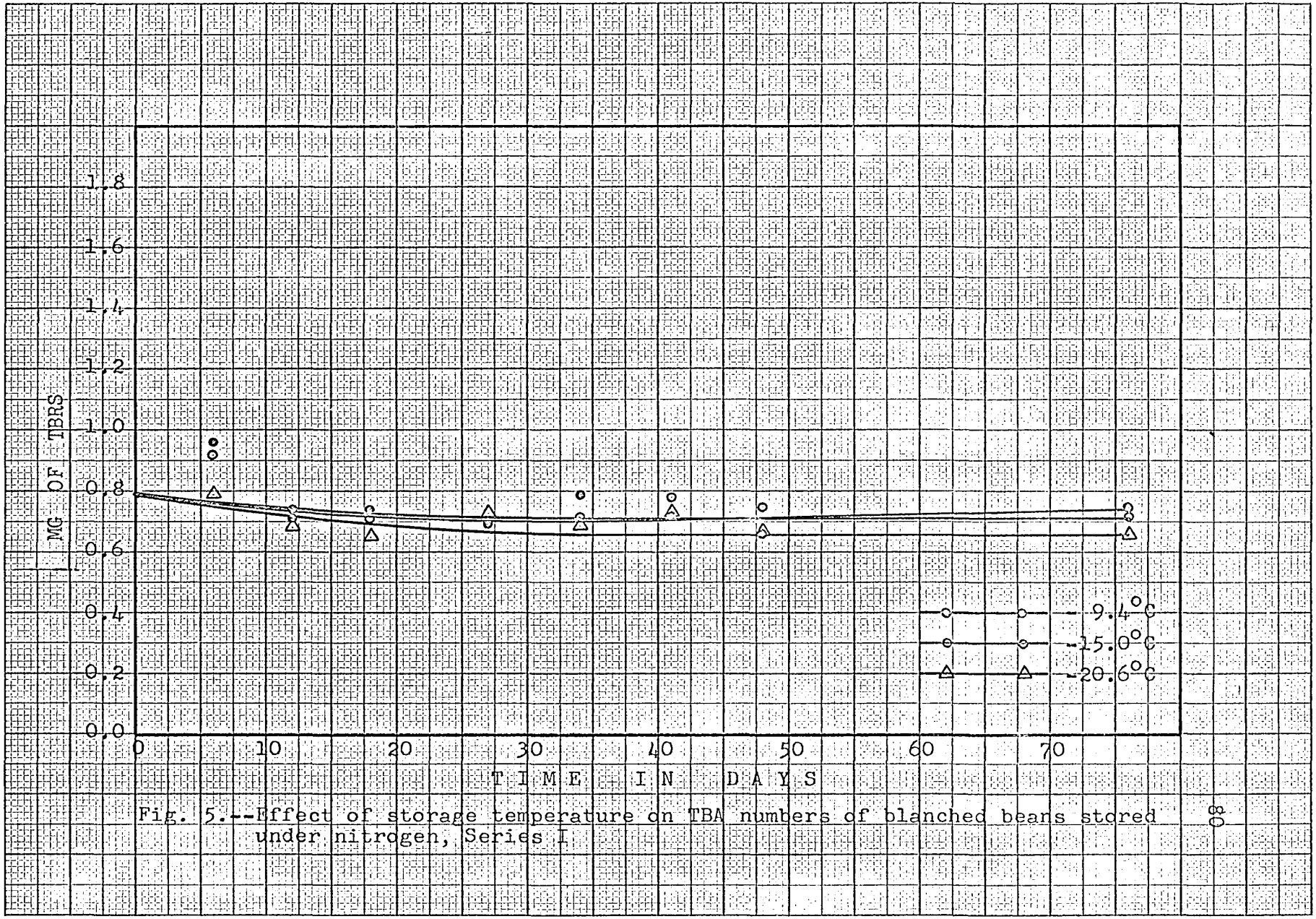

Fig. 5.--Effect of storage temperature on TBA numbers of blanched beans stored under nitrogen, Series I

TABLE 6

EFFECT OF STORAGE TEMPERATURE ON TBA NUMBERS AND FLAVOUR OF UNBLANCHED BEANS STORED UNDER AIR, SERIES I

Storage temperature °C	Storage time in days												
	Initial	3	6	9	12	15	18	21	27	34	41	48	76
- 9.4	1.00A	1.78A	1.31A	1.42A	1.14A	1.07A	1.26B	1.26B	1.26B	1.37C	1.43D	1.44D	1.46D
-15.0	1.00A	2.21A	1.40A	1.14A	1.09A	0.99A	1.23B	1.25B	1.27C	1.30D	1.32D	1.30D	1.34D
-20.6	1.00A	2.11A	1.42A	1.31A	1.16A	1.04A	1.14B	1.15B	1.20C	1.18C	1.19C	1.18C	1.16C

Flavour scores: A = good; B = slightly off; C = off; D = very off

TABLE 7

EFFECT OF STORAGE TEMPERATURE ON TBA NUMBERS AND FLAVOUR OF BLANCHED BEANS STORED UNDER AIR, SERIES I

Storage temperature °C	Storage time in days								
	Initial	6	12	18	27	34	41	48	76
- 9.4	0.78A	0.93A	0.71A	0.77A	0.56A	0.79A	0.67A	0.68A	0.79A
-15.0	0.78A	0.86A	0.74A	0.69A	0.69A	0.68A	0.77A	0.73A	0.78A
-20.6	0.78A	0.99A	0.75A	0.71A	0.76A	0.67A	0.67A	0.73A	0.67A

Flavour scores: A = good; B = slightly off; C = off; D = very off

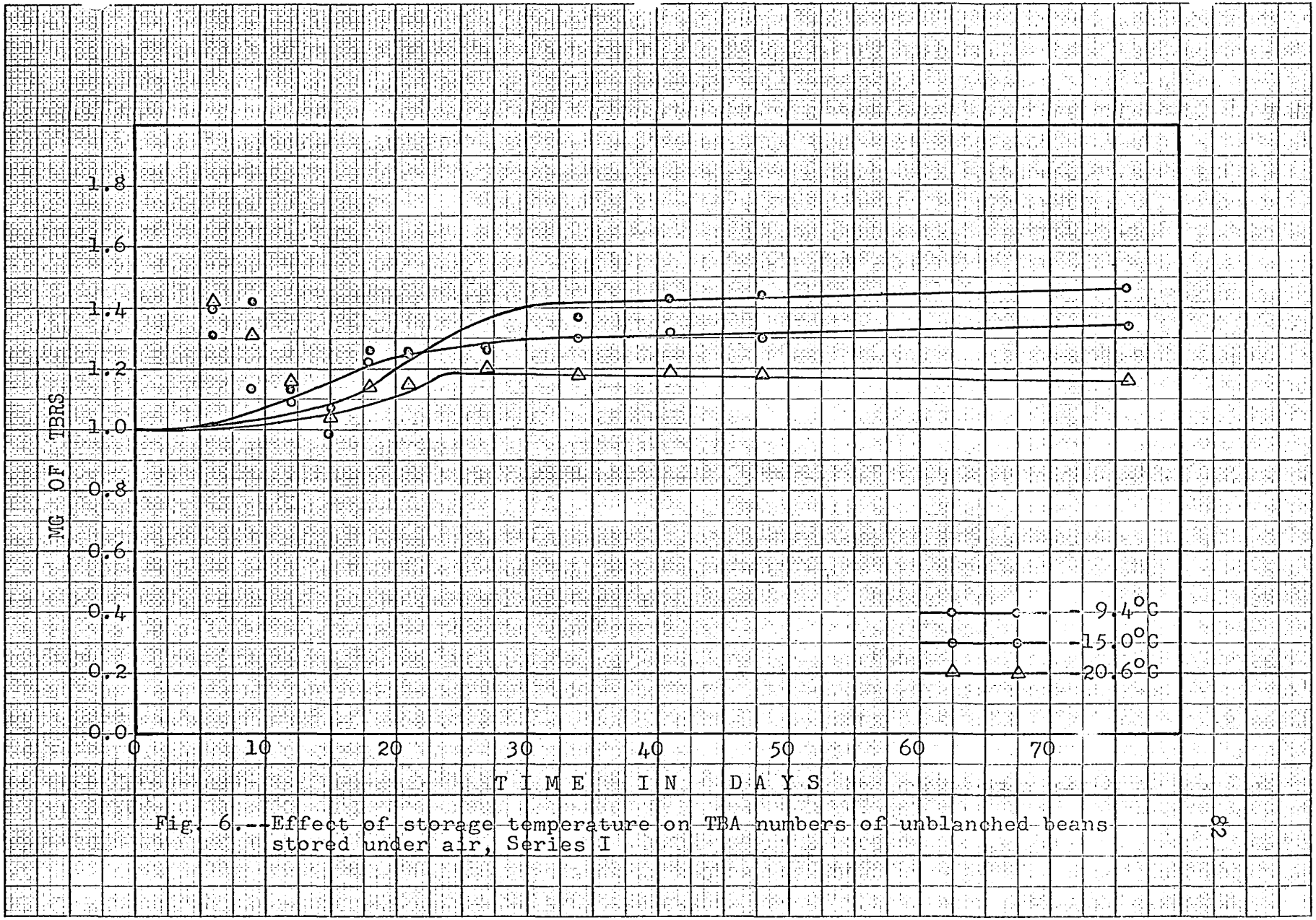

Fig. 6.--Effect of storage temperature on TBA numbers of unblanched beans stored under air, Series I

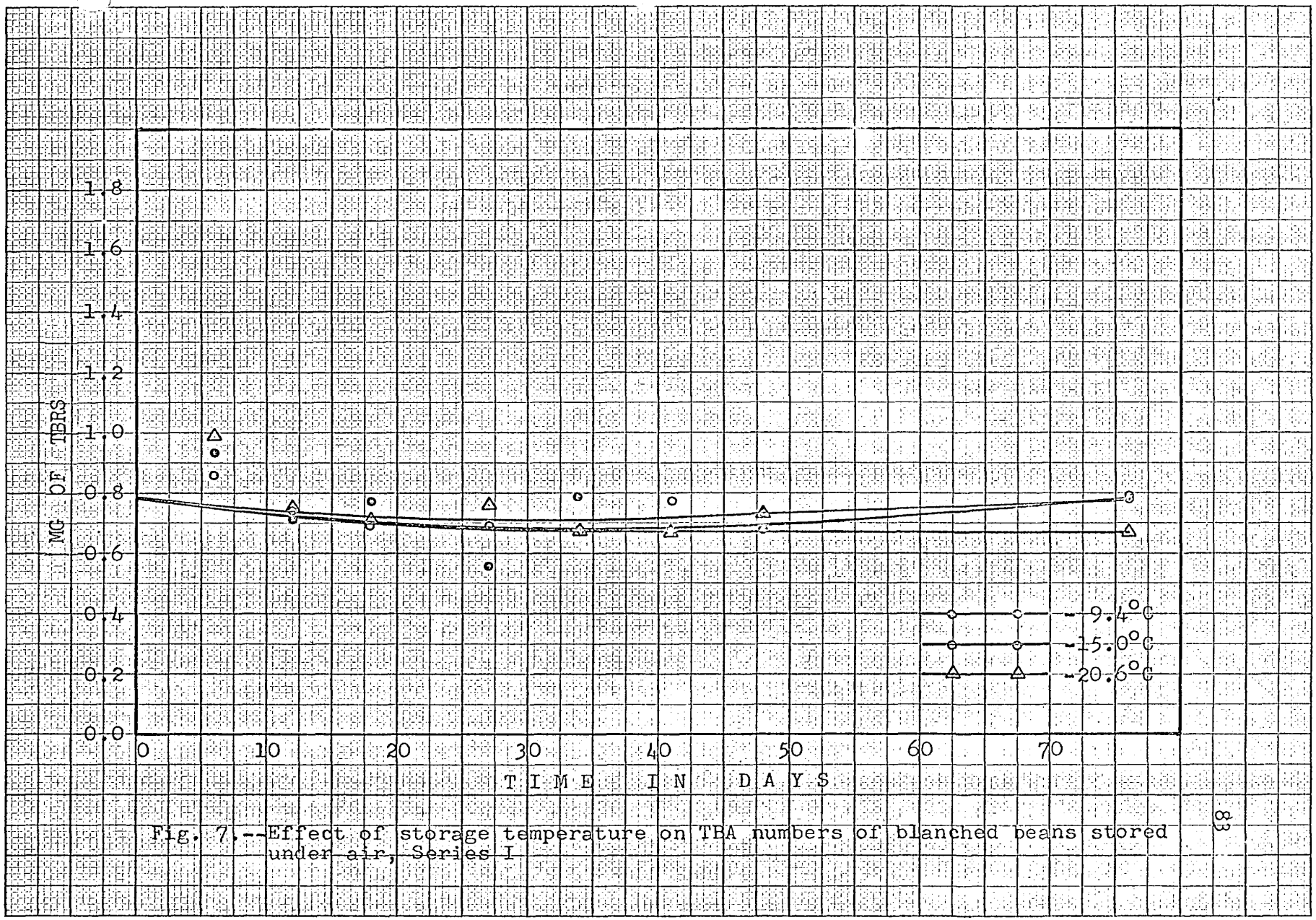

Fig. 7.--Effect of storage temperature on TBA numbers of blanched beans stored under air, Series I

mixing of the reagents before taking the spectrophotometer readings, with the result that these readings were taken on the more densely coloured solution at the bottom of the colourimeter tubes. Thus while the 6- and 9-day values for Series I are shown they were not used in plotting the results. A comparison of the results for oxygen, nitrogen and air is found in Table 20 and plotted in Figure 20.

In Series II and Series III the TBA test was used for the analysis of blanched and unblanched beans stored under oxygen, nitrogen and air at -9.4°, -15.0° and -20.6°C. The results are reported in Tables 8, 9, 10, 11, 12 and 13, and Tables 14, 15, 16, 17, 18 and 19, for Series II and Series III, respectively, and plotted in Figures 8, 9, 10, 11, 12 and 13, and Figures 14, 15, 16, 17, 18 and 19, for Series II and Series III, respectively. A comparison of the results for oxygen, nitrogen and air is found in Table 21 and in Figure 21 for Series II, and in Table 22 and in Figure 22 for Series III.

TABLE 8

EFFECT OF STORAGE TEMPERATURE ON TBA NUMBERS AND FLAVOUR OF UNBLANCHED BEANS STORED UNDER OXYGEN, SERIES II

Storage temperature °C	Storage time in days										
	Initial	3	6	9	12	15	18	21	28	35	42
- 9.4	0.95A	0.97A	1.17B	1.28C	1.38D	1.45D	1.53D	1.53D	1.54D	1.52D	1.52D
-15.0	0.95A	0.95A	0.99A	1.13B	1.20C	1.33D	1.34D	1.35D	1.34D	1.29D	1.32D
-20.6	0.95A	0.96A	0.88A	0.97A	1.05A	1.15B	1.26B	1.21B	1.25C	1.24C	1.24C

Flavour scores: A = good; B = slightly off; C = off; D = very off

TABLE 9

EFFECT OF STORAGE TEMPERATURE ON TBA NUMBERS AND FLAVOUR OF BLANCHED BEANS STORED UNDER OXYGEN, SERIES II

Storage temperature °C	Storage time in days						
	Initial	6	12	18	28	35	42
- 9.4	0.65A	0.61A	0.56A	0.61A	0.60A	0.60A	0.60A
-15.0	0.65A	0.65A	0.58A	0.62A	0.62A	0.60A	0.60A
-20.6	0.65A	0.66A	0.59A	0.55A	0.60A	0.59A	0.59A

Flavour scores: A = good; B = slightly off; C = off; D = very off

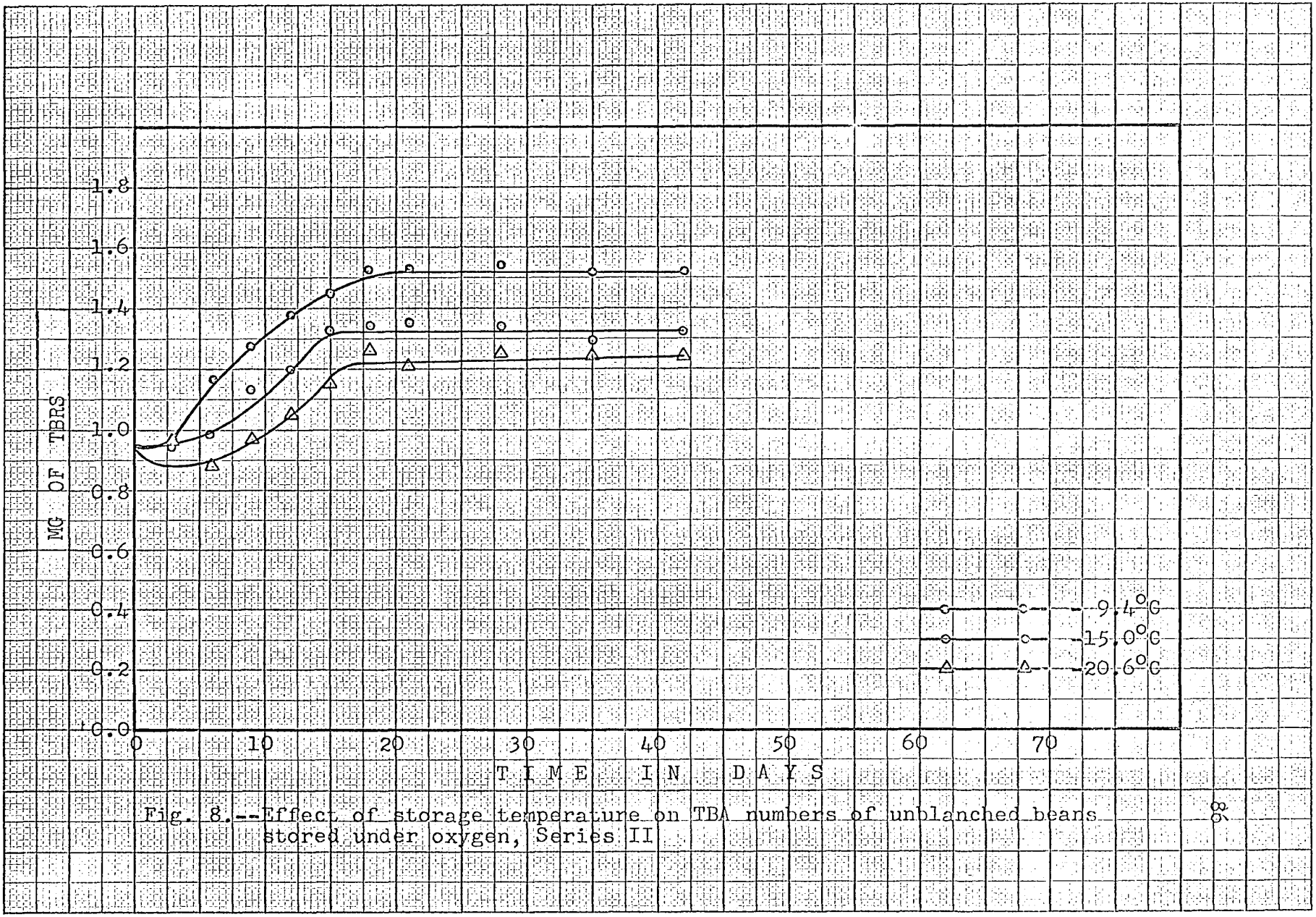

Fig. 8.--Effect of storage temperature on TBA numbers of unblanched beans stored under oxygen, Series II

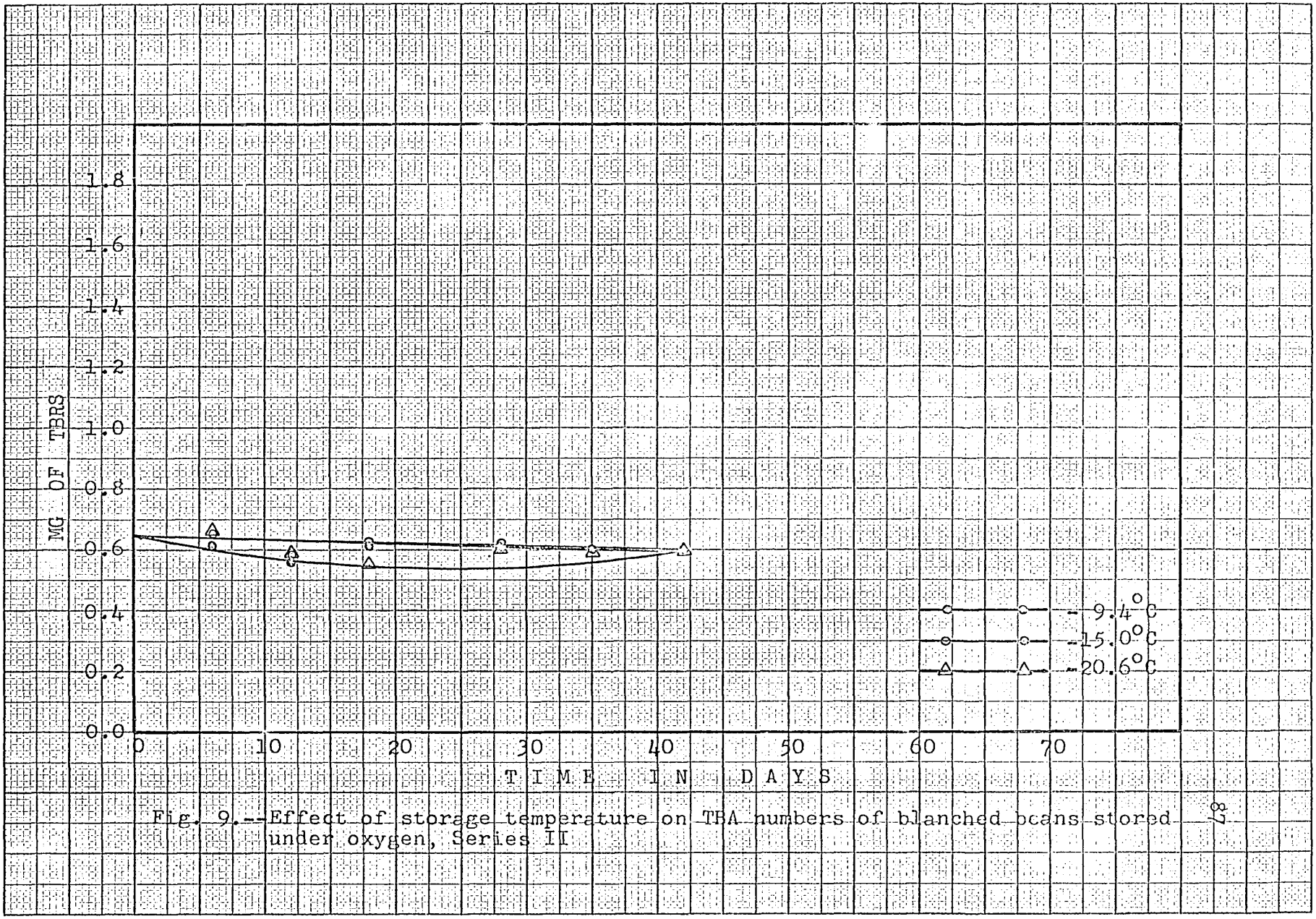

Fig. 9.--Effect of storage temperature on TBA numbers of blanched beans stored under oxygen, Series II

TABLE 10

EFFECT OF STORAGE TEMPERATURE ON TBA NUMBERS AND FLAVOUR OF UNBLANCHED BEANS STORED UNDER NITROGEN, SERIES II

Storage temperature °C	Storage time in days										
	Initial	3	6	9	12	15	18	21	28	35	42
- 9.4	0.95A	0.84A	0.99A	0.95A	0.95A	1.01A	1.17B	1.21B	1.24C	1.21C	1.24C
-15.0	0.95A	0.88A	0.87A	0.87A	0.90A	0.94A	1.03A	1.02A	1.05A	1.14B	1.15B
-20.6	0.95A	0.87A	0.86A	0.84A	0.85A	0.87A	0.96A	0.99A	0.97A	1.00A	1.04A

Flavour scores: A = good; B = slightly off; C = off; D = very off

TABLE 11

EFFECT OF STORAGE TEMPERATURE ON TBA NUMBERS AND FLAVOUR OF BLANCHED BEANS STORED UNDER NITROGEN, SERIES II

Storage temperature °C	Storage time in days						
	Initial	6	12	18	28	35	42
- 9.4	0.65A	0.63A	0.59A	0.61A	0.58A	0.58A	0.58A
-15.0	0.65A	0.66A	0.60A	0.62A	0.53A	0.56A	0.54A
-20.6	0.65A	0.68A	0.59A	0.62A	0.60A	0.60A	0.61A

Flavour scores: A = good; B = slightly off; C = off; D = very off

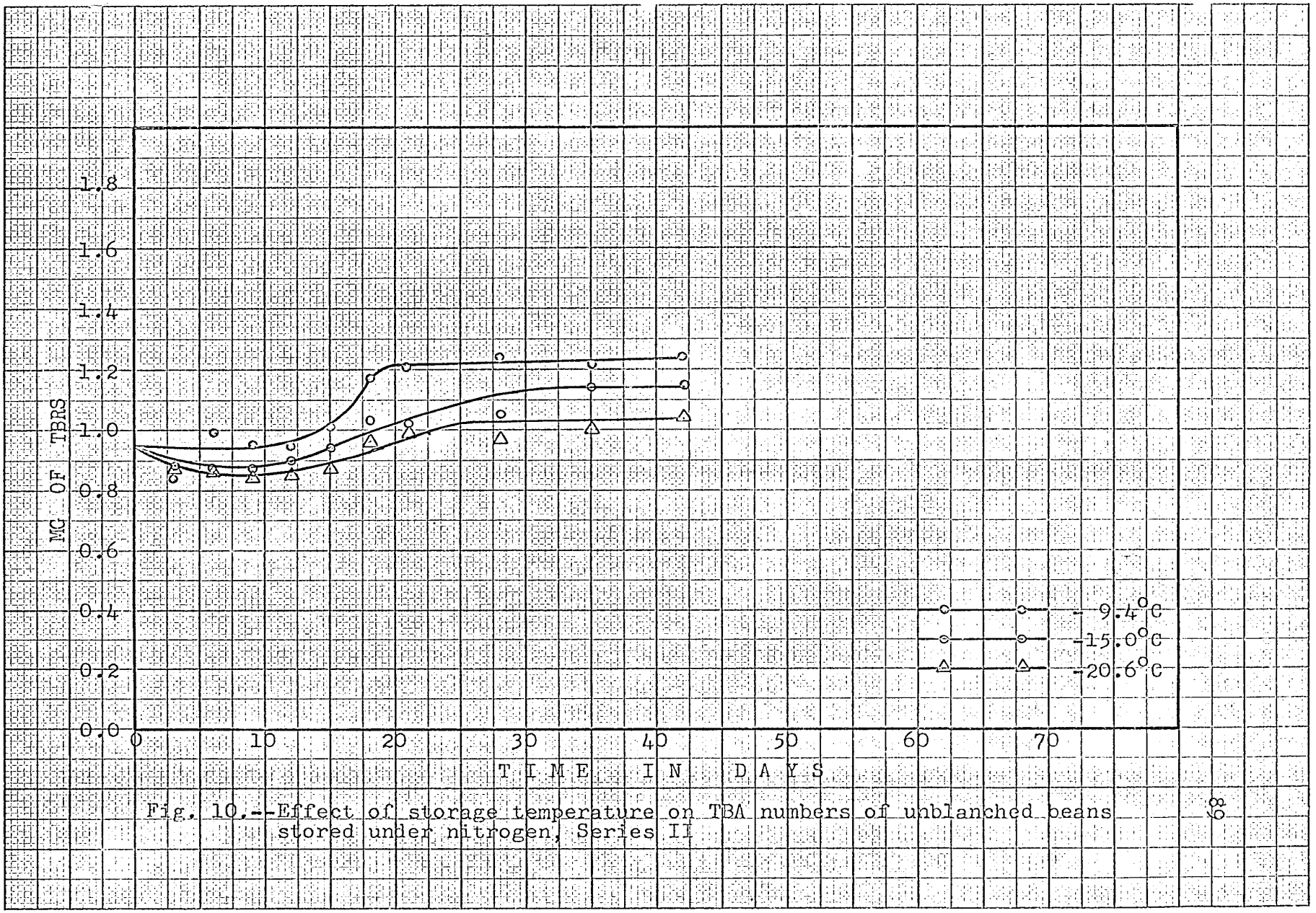

Fig. 10.--Effect of storage temperature on TBA numbers of unblanched beans stored under nitrogen, Series II

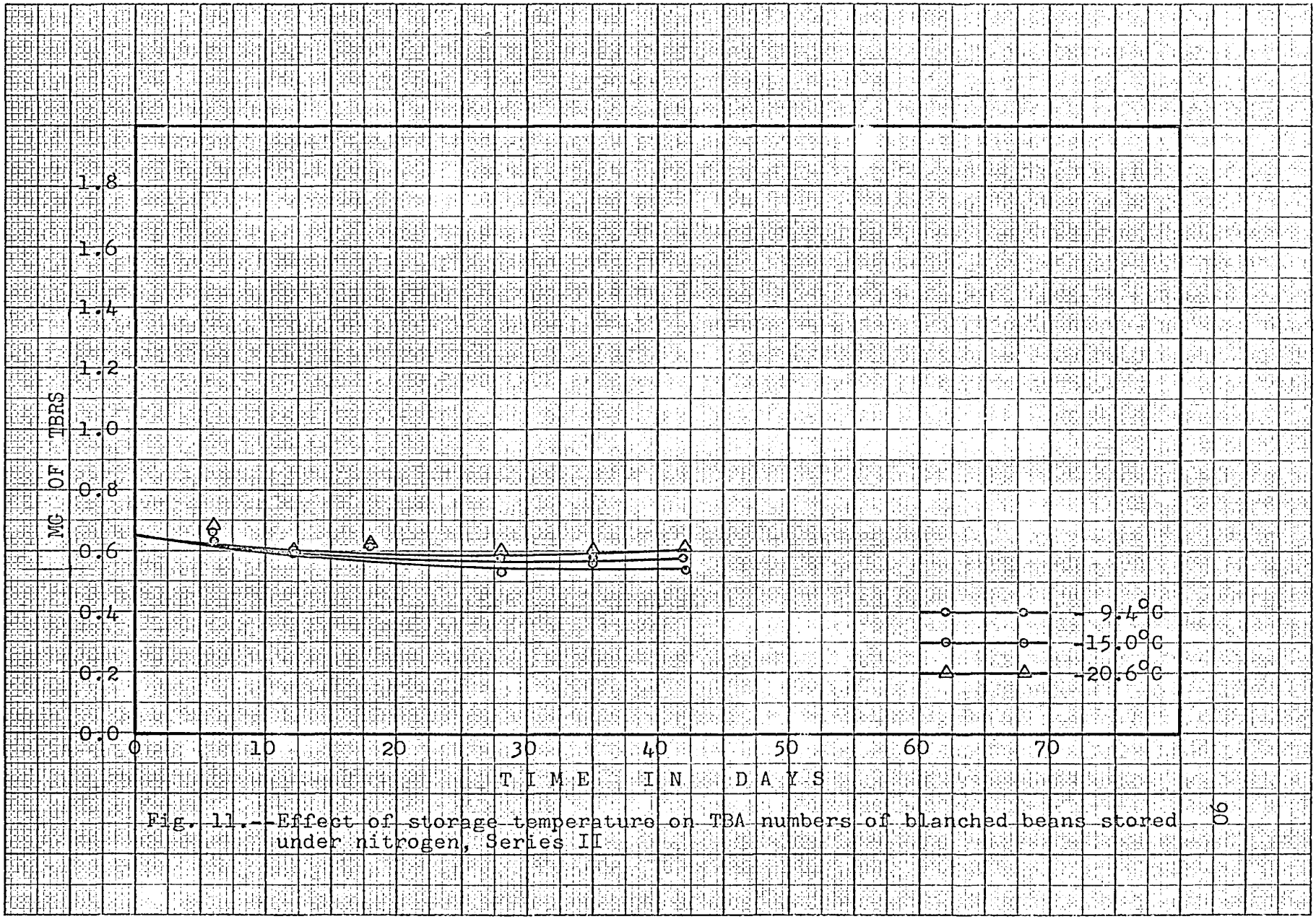

Fig. 11.--Effect of storage temperature on TBA numbers of blanched beans stored under nitrogen, Series II

TABLE 12

EFFECT OF STORAGE TEMPERATURE ON TBA NUMBERS AND FLAVOUR OF UNBLANCHED BEANS STORED UNDER AIR, SERIES II

Storage temperature °C	Storage time in days										
	Initial	3	6	9	12	15	18	21	28	35	42
- 9.4	0.95A	0.92A	0.96A	0.99A	1.05A	1.01A	1.20B	1.24C	1.28D	1.32D	1.33D
-15.0	0.95A	0.91A	1.02A	0.99A	0.99A	1.00A	1.05A	1.14B	1.11B	1.12C	1.13C
-20.6	0.95A	0.90A	0.88A	0.87A	0.85A	0.91A	0.91A	0.97A	0.97A	0.94A	0.95A

Flavour scores: A = good; B = slightly off; C = off; D = very off

TABLE 13

EFFECT OF STORAGE TEMPERATURE ON TBA NUMBERS AND FLAVOUR OF BLANCHED BEANS STORED UNDER AIR, SERIES II

Storage temperature °C	Storage time in days						
	Initial	6	12	18	28	35	42
- 9.4	0.65A	0.73A	0.60A	0.63A	0.61A	0.61A	0.61A
-15.0	0.65A	0.67A	0.58A	0.65A	0.61A	0.54A	0.59A
-20.6	0.65A	0.66A	0.59A	0.63A	0.56A	0.55A	0.55A

Flavour scores: A = good; B = slightly off; C = off; D = very off

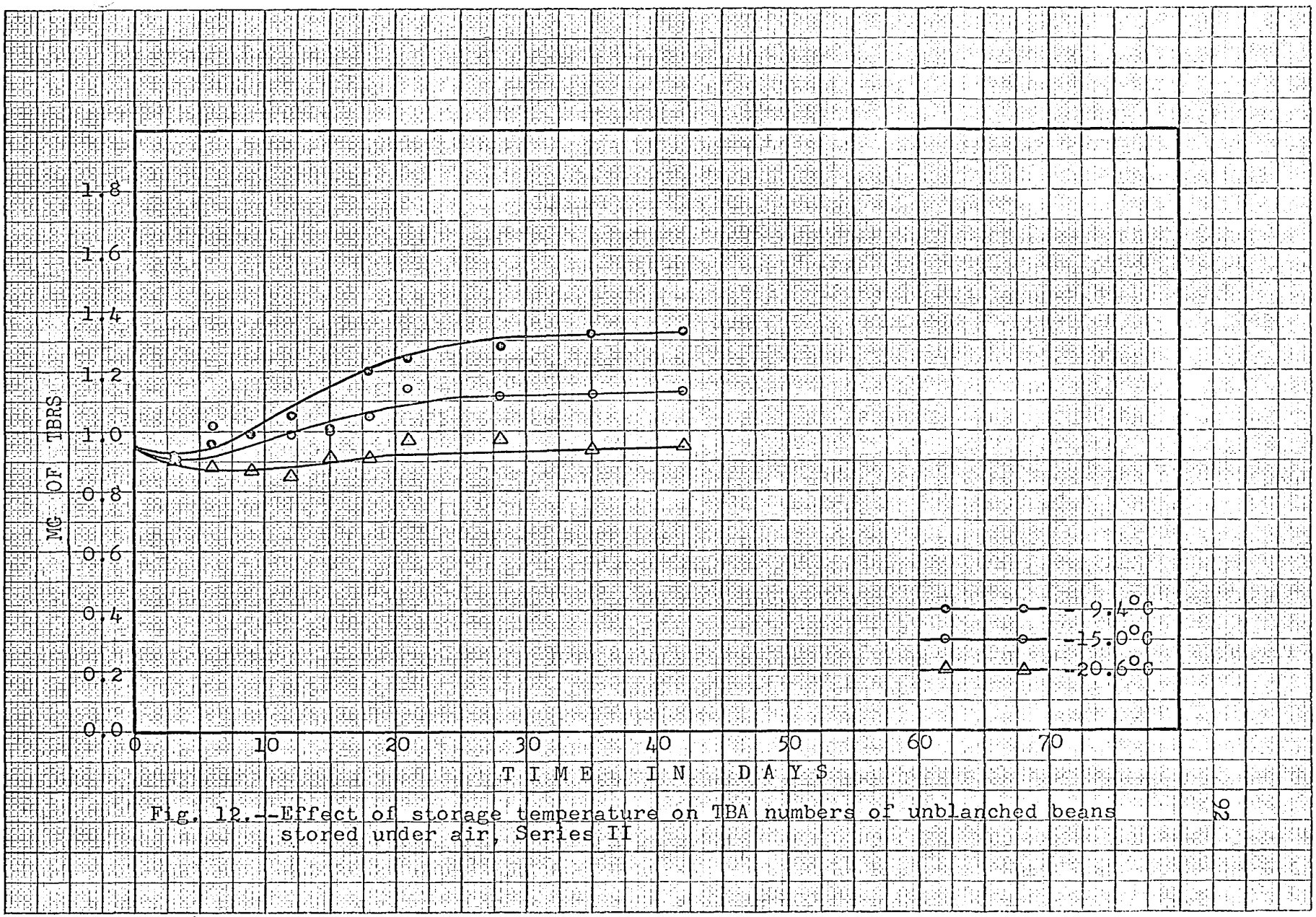

Fig. 12.--Effect of storage temperature on TBA numbers of unblanched beans stored under air, Series II

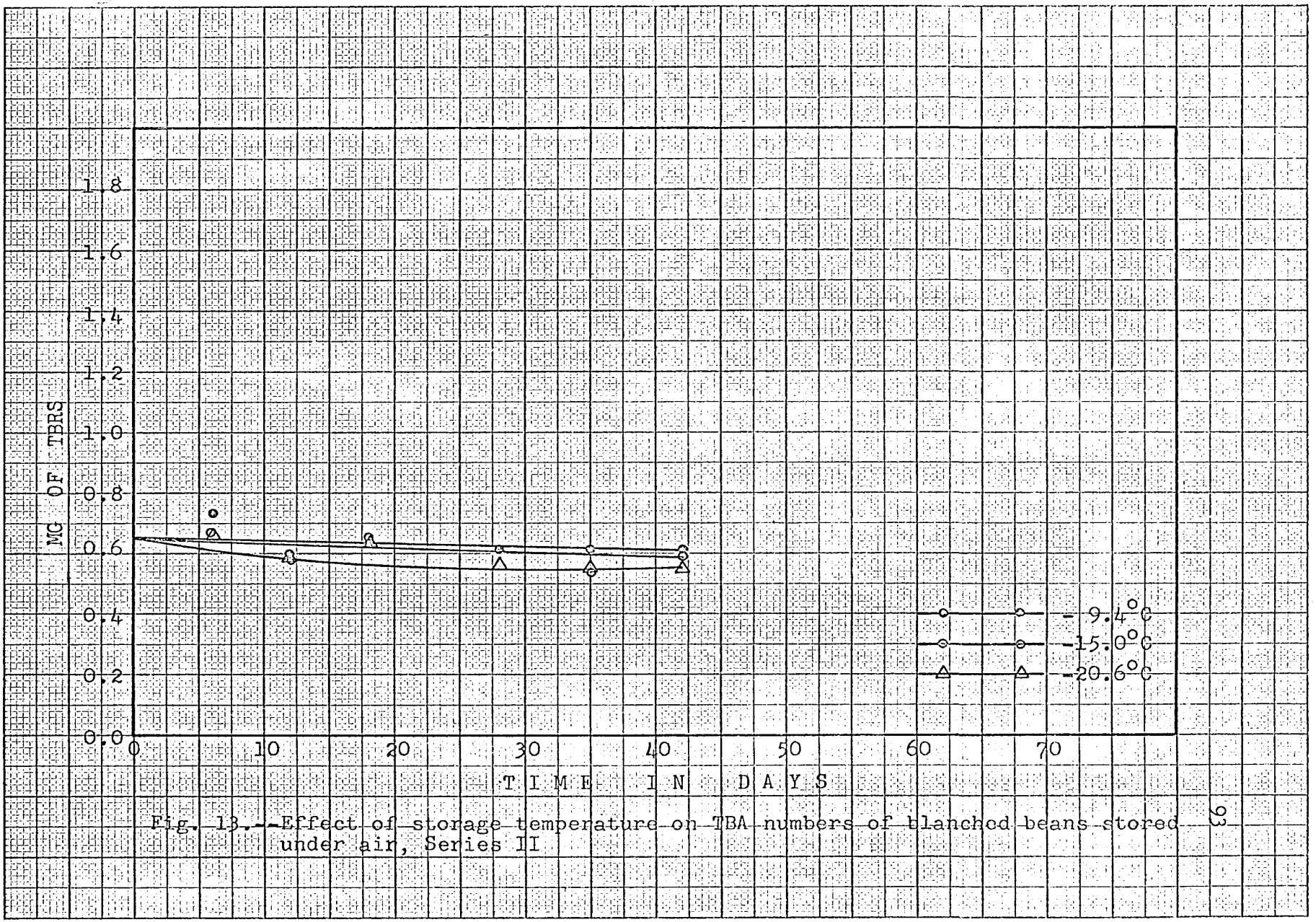

Fig. 13.--Effect of storage temperature on TBA numbers of blanched beans stored under air, Series II

TABLE 14

EFFECT OF STORAGE TEMPERATURE ON TBA NUMBERS AND FLAVOUR OF UNBLANCHED BEANS STORED UNDER OXYGEN, SERIES III

Storage temperature °C	Storage time in days										
	Initial	3	6	9	12	15	18	21	28	35	42
- 9.4	0.95A	0.95A	1.10B	1.18B	1.25C	1.31D	1.36D	1.38D	1.51D	1.49D	1.49D
-15.0	0.95A	0.99A	1.06A	1.12B	1.16B	1.26C	1.31D	1.33D	1.37D	1.37D	1.37D
-20.6	0.95A	0.96A	1.01A	1.01A	1.07A	1.12B	1.21C	1.26C	1.21C	1.24C	1.24C

Flavour scores: A = good; B = slightly off; C = off; D = very off

TABLE 15

EFFECT OF STORAGE TEMPERATURE ON TBA NUMBERS AND FLAVOUR OF BLANCHED BEANS STORED UNDER OXYGEN, SERIES III

Storage temperature °C	Storage time in days						
	Initial	6	12	18	28	35	42
- 9.4	0.65A	0.61A	0.56A	0.61A	0.60A	0.60A	0.60A
-15.0	0.65A	0.65A	0.58A	0.62A	0.62A	0.60A	0.60A
-20.6	0.65A	0.66A	0.59A	0.55A	0.60A	0.59A	0.59A

Flavour scores: A = good; B = slightly off; C = off; D = very off

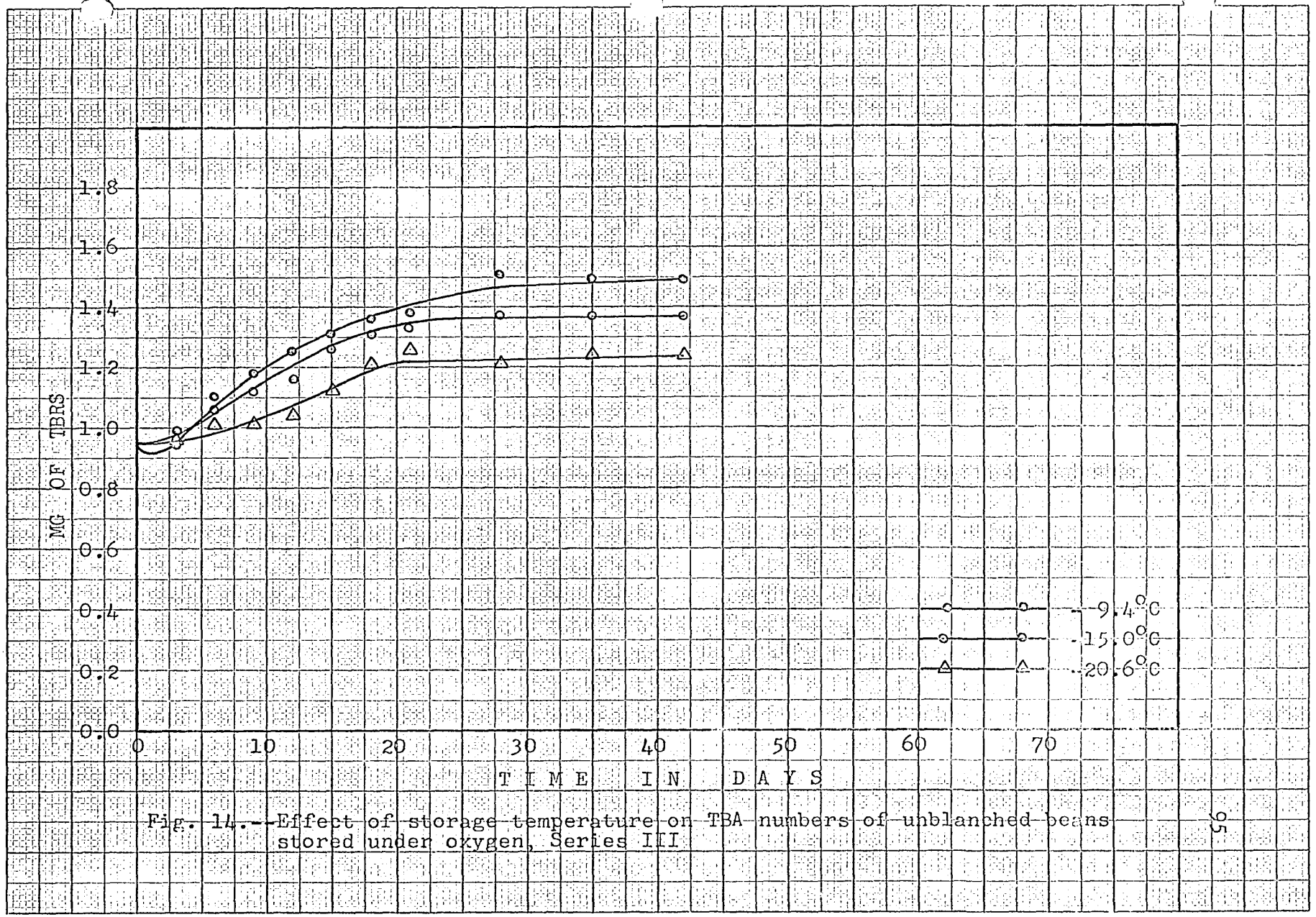

Fig. 14.--Effect of storage temperature on TBA numbers of unblanched beans stored under oxygen, Series III

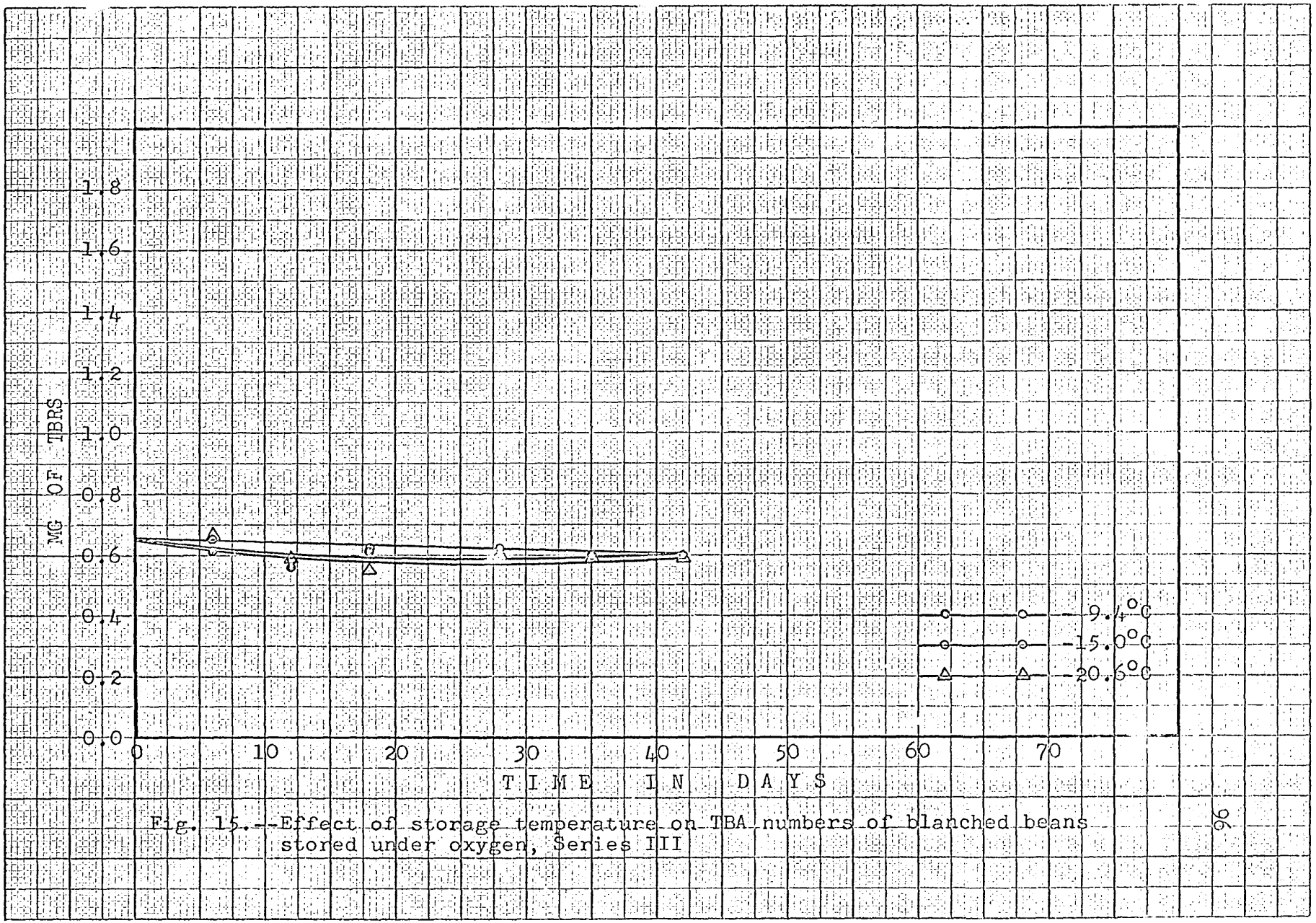

Fig. 15.--Effect of storage temperature on TBA numbers of blanched beans stored under oxygen, Series III

TABLE 16

EFFECT OF STORAGE TEMPERATURE ON TBA NUMBERS AND FLAVOUR OF UNBLANCHED BEANS STORED UNDER NITROGEN, SERIES III

Storage temperature °C	Storage time in days										
	Initial	3	6	9	12	15	18	21	28	35	42
- 9.4	0.95A	0.93A	0.91A	0.92A	1.04A	1.00A	1.15B	1.20C	1.21C	1.27C	1.27C
-15.0	0.95A	0.88A	0.91A	0.95A	1.00A	1.00A	1.03A	1.02A	1.16B	1.19B	1.19B
-20.6	0.95A	0.88A	0.87A	0.94A	0.94A	0.94A	1.02A	1.03A	1.09A	1.04A	1.04A

Flavour scores: A = good; B = slightly off; C = off; D = very off

TABLE 17

EFFECT OF STORAGE TEMPERATURE ON TBA NUMBERS AND FLAVOUR OF BLANCHED BEANS STORED UNDER NITROGEN, SERIES III

Storage temperature °C	Storage time in days						
	Initial	6	12	18	28	35	42
- 9.4	0.65A	0.63A	0.59A	0.61A	0.58A	0.58A	0.58A
-15.0	0.65A	0.66A	0.60A	0.62A	0.53A	0.56A	0.54A
-20.6	0.65A	0.68A	0.59A	0.62A	0.60A	0.60A	0.61A

Flavour scores: A = good; B = slightly off; C = off; D = very off

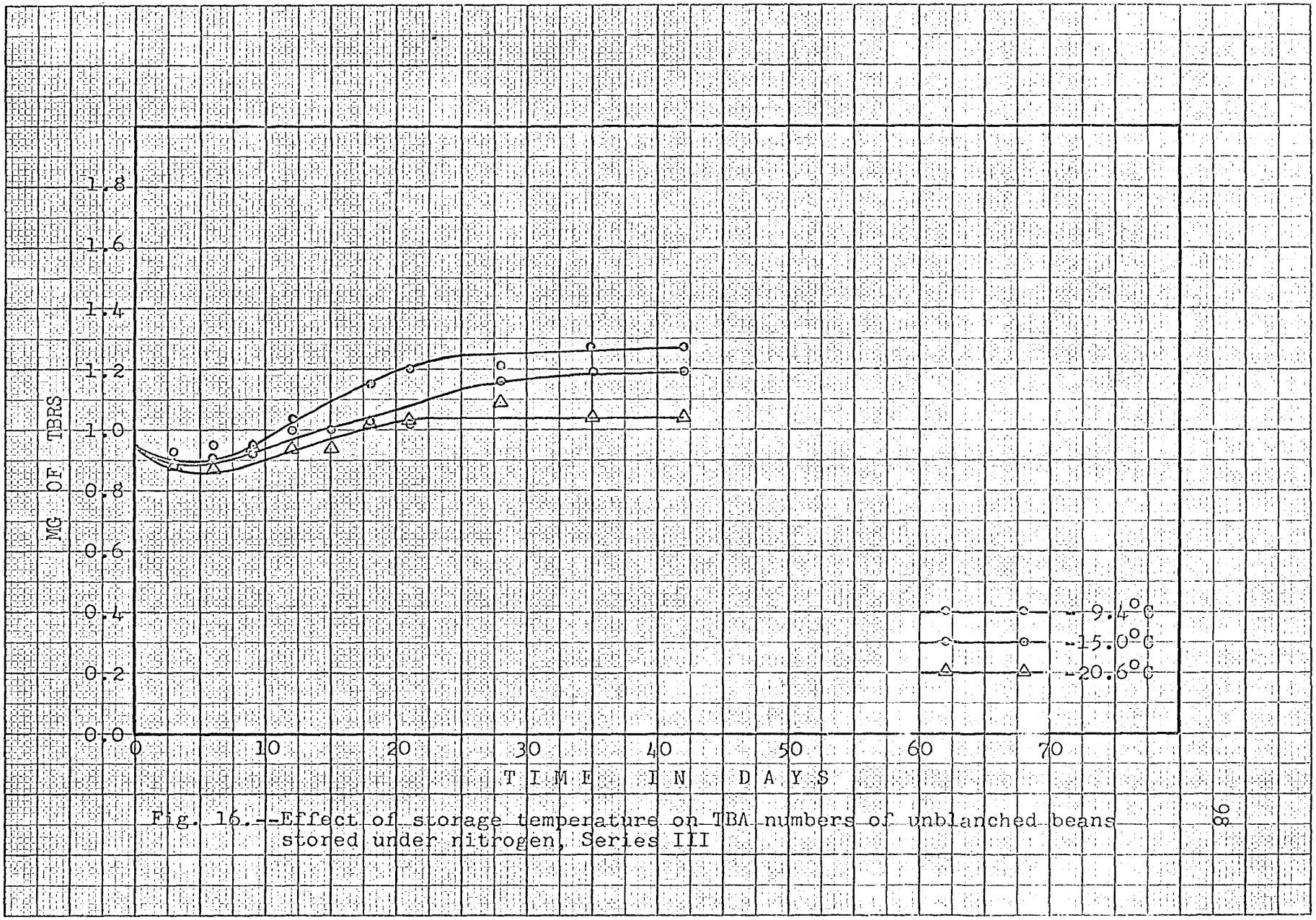

Fig. 16.--Effect of storage temperature on TBA numbers of unblanched beans stored under nitrogen, Series III

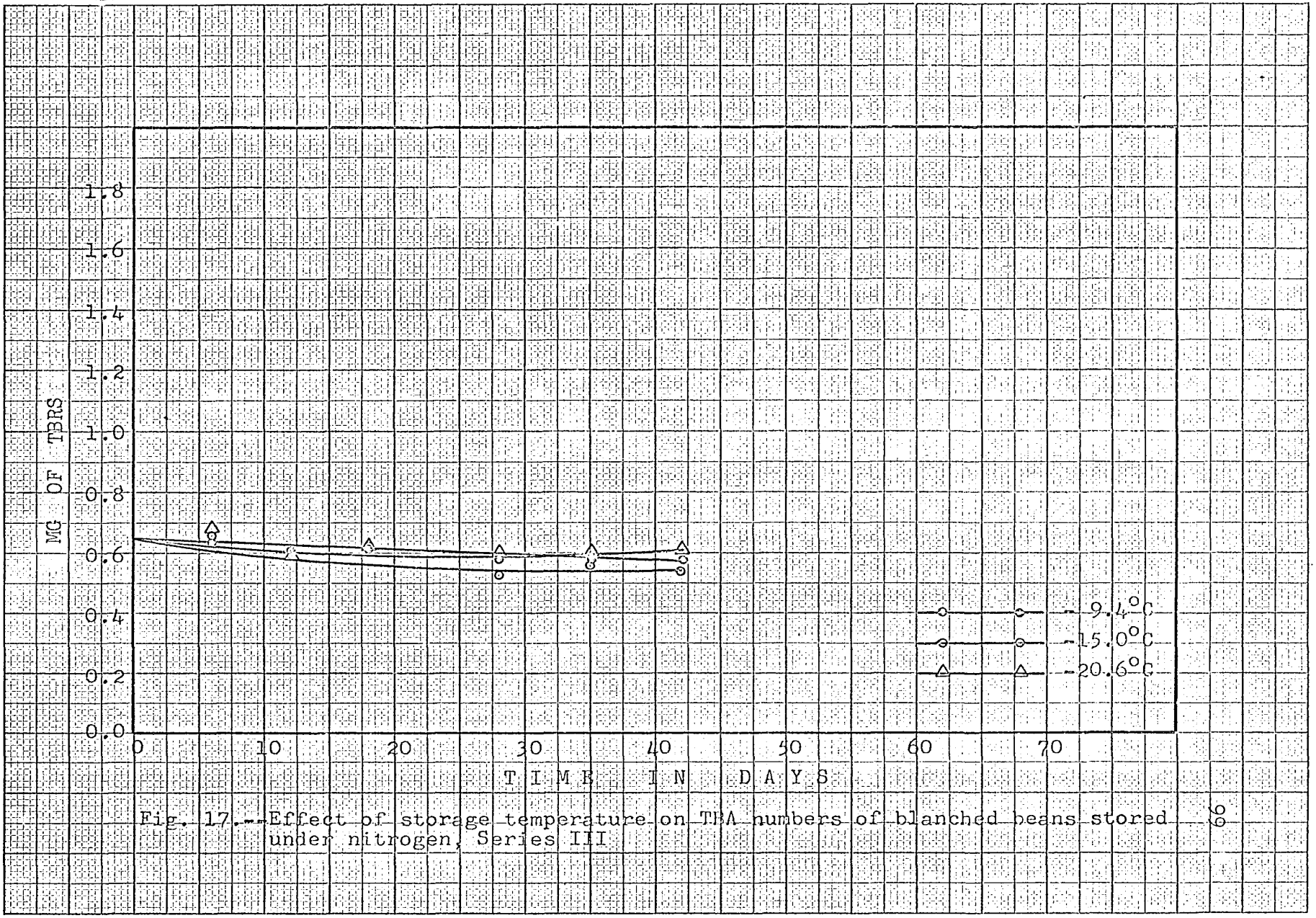

Fig. 17.--Effect of storage temperature on TBA numbers of blanched beans stored under nitrogen, Series III

TABLE 18

EFFECT OF STORAGE TEMPERATURE ON TBA NUMBERS AND FLAVOUR OF UNBLANCHED BEANS STORED UNDER AIR, SERIES III

Storage temperature °C	Storage time in days										
	Initial	3	6	9	12	15	18	21	28	35	42
- 9.4	0.95A	0.91A	1.00A	1.00A	1.15B	1.22C	1.25C	1.27C	1.28C	1.30D	1.31D
-15.0	0.95A	0.86A	0.97A	0.95A	1.02A	1.04A	1.10B	1.09B	1.19C	1.19C	1.19C
-20.6	0.95A	0.92A	0.96A	0.99A	0.99A	0.97A	1.00A	0.99A	1.01A	1.02A	1.02A

Flavour scores: A = good; B = slightly off; C = off; D = very off

TABLE 19

EFFECT OF STORAGE TEMPERATURE ON TBA NUMBERS AND FLAVOUR OF BLANCHED BEANS STORED UNDER AIR, SERIES III

Storage temperature °C	Storage time in days						
	Initial	6	12	18	28	35	42
- 9.4	0.65A	0.73A	0.60A	0.63A	0.61A	0.61A	0.61A
-15.0	0.65A	0.67A	0.58A	0.65A	0.61A	0.54A	0.59A
-20.6	0.65A	0.66A	0.59A	0.63A	0.56A	0.55A	0.55A

Flavour scores: A = good; B = slightly off; C = off; D = very off

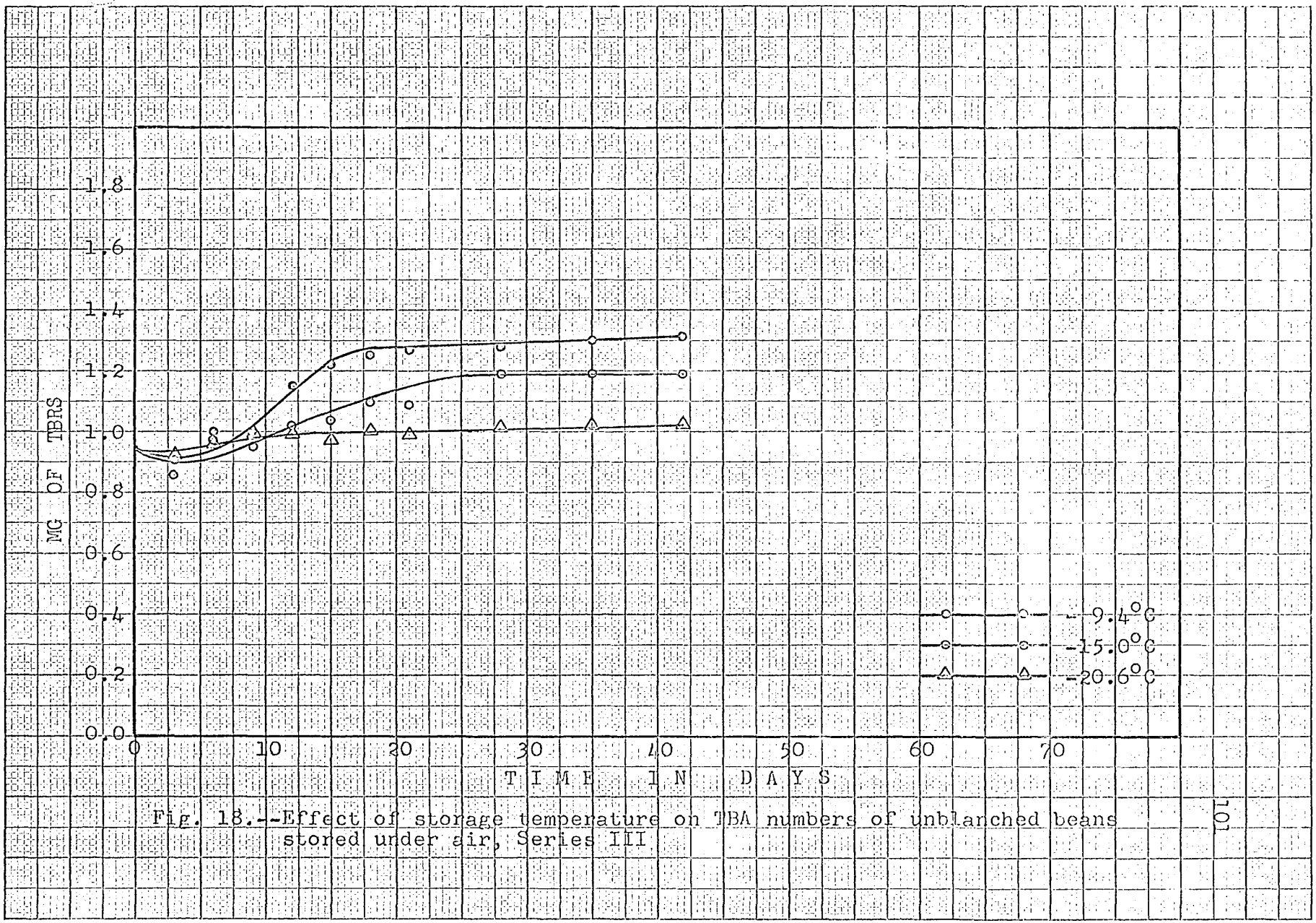

Fig. 18.--Effect of storage temperature on TBA numbers of unblanched beans stored under air, Series III

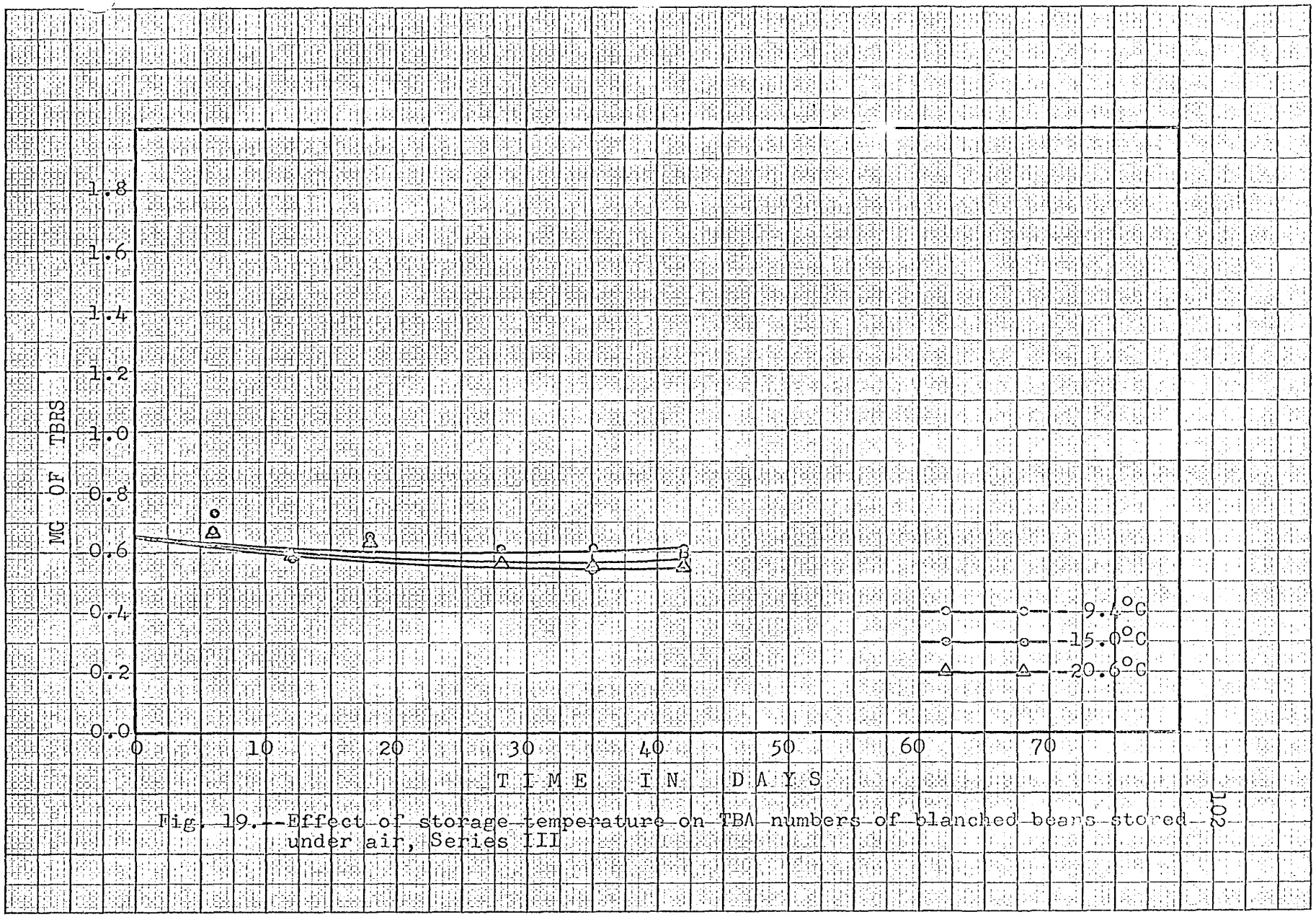

Fig. 19.--Effect of storage temperature on TBA numbers of blanched beans stored under air, Series III

TABLE 20

COMPARISON OF TBA NUMBERS AND FLAVOUR OF UNBLANCHED BEANS STORED UNDER OXYGEN, NITROGEN, AND AIR AT -9.4°C, SERIES I

Storage atmosphere	Storage time in days												
	Initial	3	6	9	12	15	18	21	27	34	41	48	76
Oxygen	1.00A	2.18A	1.55A	1.64A	1.47A	1.52B	1.54C	1.53C	1.53D	1.51D	1.51D	1.56D	1.57D
Nitrogen	1.00A	1.83A	1.32A	1.71A	0.93A	1.04A	1.37B	1.38B	1.37B	1.37B	1.38C	1.40D	1.40D
Air	1.00A	1.78A	1.31A	1.42A	1.14A	1.07A	1.26B	1.26B	1.26B	1.37C	1.43D	1.44D	1.46D

Flavour scores: A = good; B = slightly off; C = off; D = very off

TABLE 21

COMPARISON OF TBA NUMBERS AND FLAVOUR OF UNBLANCHED BEANS STORED UNDER OXYGEN, NITROGEN, AND AIR AT -9.4°C, SERIES II

Storage atmosphere	Storage time in days										
	Initial	3	6	9	12	15	18	21	28	35	42
Oxygen	0.95A	0.97A	1.17B	1.28C	1.38D	1.45D	1.53D	1.53D	1.54D	1.52D	1.52D
Nitrogen	0.95A	0.84A	0.99A	0.95A	0.95A	1.01A	1.17B	1.21B	1.24C	1.21C	1.24C
Air	0.95A	0.92A	0.96A	0.99A	1.05A	1.01A	1.20B	1.24C	1.28D	1.32D	1.33D

Flavour scores: A = good; B = slightly off; C = off; D = very off

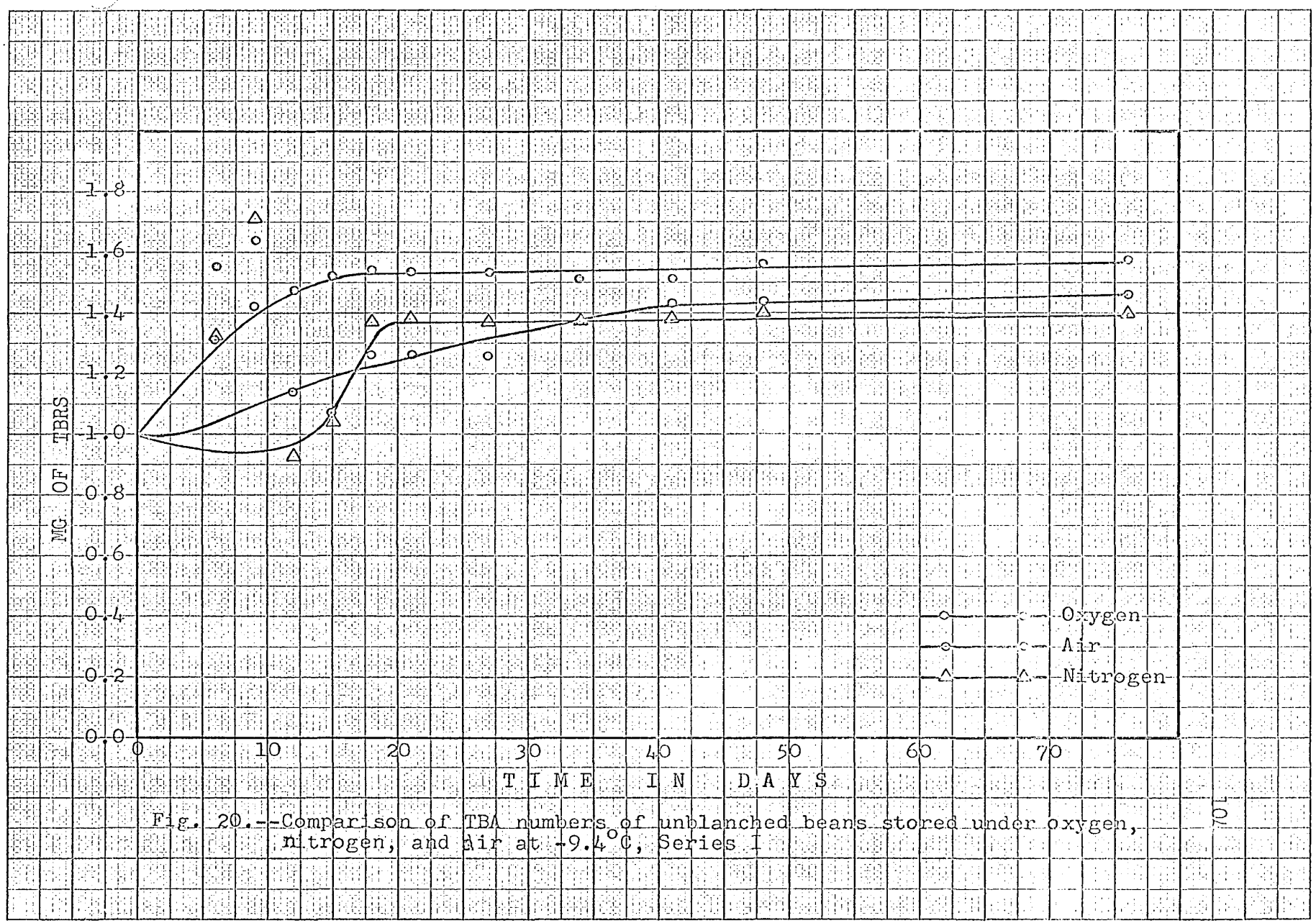

Fig. 20.--Comparison of TBA numbers of unblanched beans stored under oxygen, nitrogen, and air at -9.4°C, Series I

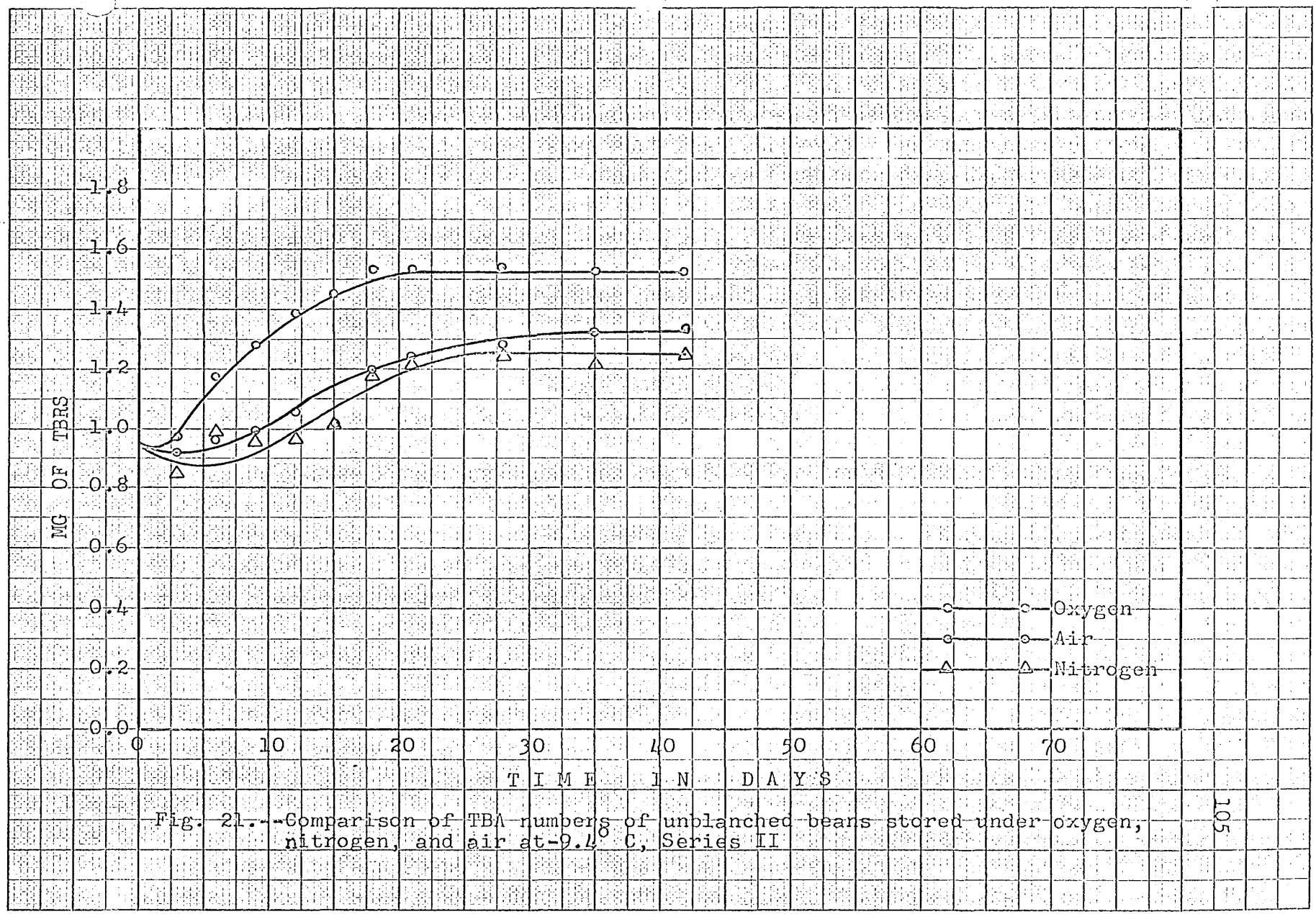

Fig. 21.--Comparison of TBA numbers of unblanched beans stored under oxygen, nitrogen, and air at -9.4° C, Series II

TABLE 22

COMPARISON OF TBA NUMBERS AND FLAVOUR OF UNBLANCHED BEANS STORED UNDER OXYGEN, NITROGEN, AND AIR AT - 9.4°C, SERIES III

Storage atmosphere	Storage time in days										
	Initial	3	6	9	12	15	18	21	28	35	42
Oxygen	0.95A	0.95A	1.10B	1.18B	1.25C	1.31D	1.36D	1.38D	1.51D	1.49D	1.49D
Nitrogen	0.95A	0.93A	0.91A	0.92A	1.04A	1.00A	1.15B	1.20C	1.21C	1.27C	1.27C
Air	0.95A	0.91A	1.00A	1.00A	1.15B	1.22C	1.25C	1.27C	1.28C	1.30D	1.31D

Flavour scores: A = good; B = slightly off; C = off; D = very off

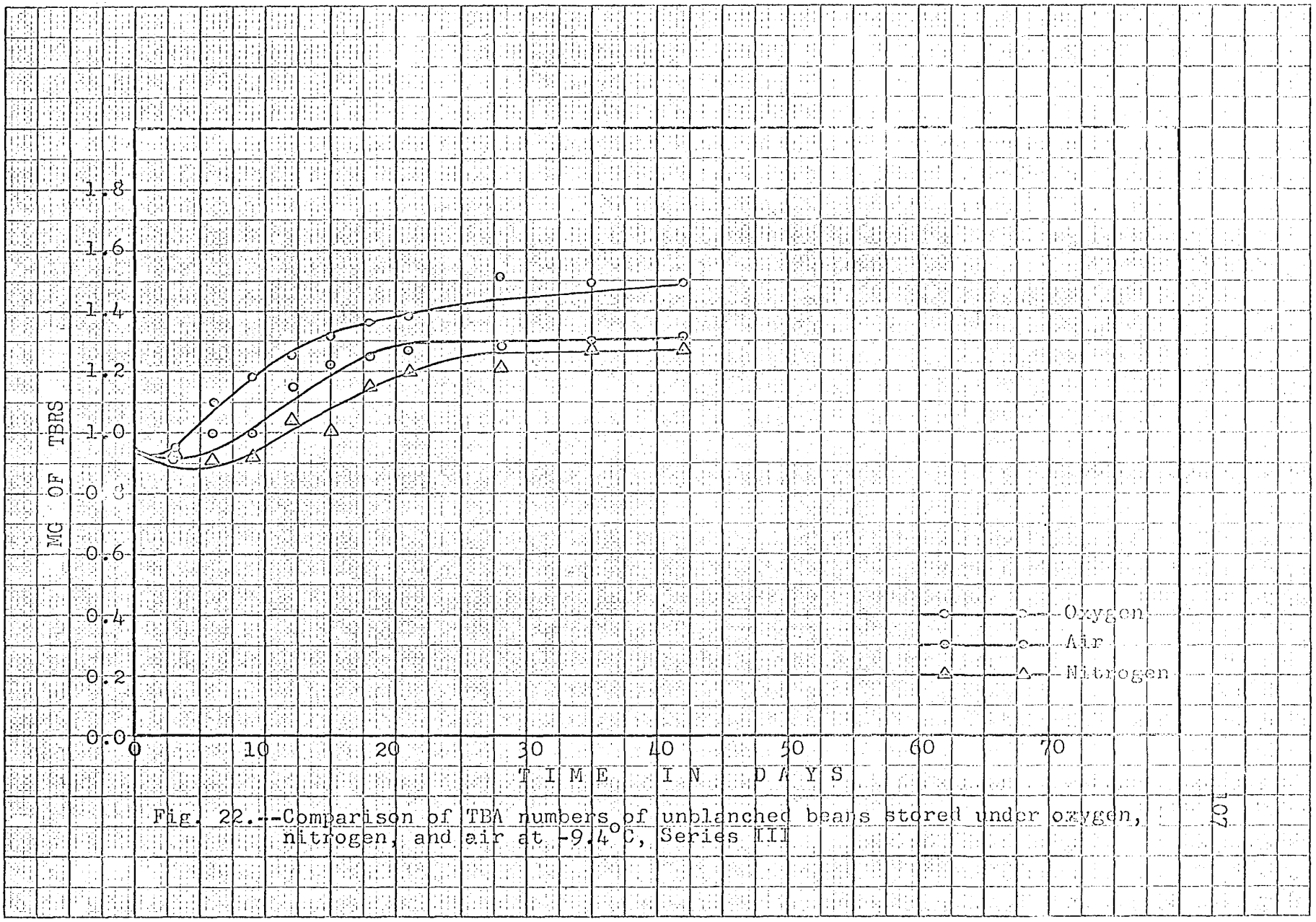

Fig. 22.--Comparison of TBA numbers of unblanched beans stored under oxygen, nitrogen, and air at -9.4°C, Series III

Effect of Blanching Upon Production of TBRS

The results of analysis on blanched and unblanched material stored under various conditions are presented in Tables 2 to 19 and Figures 2 to 19. They show that in every series the TBA numbers of raw beans increased during storage at the temperatures used while beans blanched for 3 min showed no significant increase in TBA numbers. In fact, these values remained fairly constant throughout the storage period. This clearly indicates that the TBRS accumulates as a result of the activity of certain enzymes which are inhibited by a 3-min heat treatment.

Effect of Storage Temperature Upon Production of TBRS

The effect of various storage temperatures on the production and accumulation of TBRS was studied. The results of these experiments, presented in Tables 2 to 19 and plotted in Figures 2 to 19, indicate that the greatest production of TBRS occurred at -9.4°C, the highest temperature used, in all three series. A lower production was observed in samples stored at -15.0°C. At the lowest storage temperature, -20.6°C, an even lower production was observed and, in some cases, the

amounts were not enough to indicate off-flavour development.

The highest TBRS concentrations were reached most rapidly in the unblanched samples stored at the highest storage temperature. The rate of production in raw samples was highest during the first two to three weeks of storage. After the initial rapid increase in TBRS, there was a levelling off in production with only small increases observed as the storage period progressed.

The difference between highest TBRS concentrations obtained in the three series was quite small and would be due to the use of different varieties of beans and to experimental variance.

Effect of Composition of Atmosphere in the Container Upon Production of TBRS

The atmosphere of the container had a definite effect upon the production of TBRS during storage in all three series, the effect being most noticeable at the highest storage temperature, -9.4°C. A comparison of TBA numbers obtained at this temperature, for unblanched samples stored under oxygen, nitrogen and air, is found in Tables 20 to 22 and plotted in Figures 20 to 22. The effects of the gas treatments at all temperatures are

recorded in Tables 2 to 19 and plotted in Figures 2 to 19. A comparison of the different atmospheres used indicates that TBRS accumulated in the following order: oxygen > air > nitrogen.

Thus it appears that as the concentration of oxygen in the container decreased the amount of TBRS produced decreased. In all series the production of TBRS was highest in unblanched beans stored under oxygen (aerobic conditions) and lowest in unblanched beans stored under nitrogen (anaerobic conditions). The results obtained indicate that the production of TBRS is mainly an oxidative process but can occur under anaerobic conditions.

In the blanched material in all three series, the TBA numbers obtained fluctuated only slightly and could not be attributed to effect of storage atmosphere. The fluctuations could be due to biological variations in the samples.

Off-flavour Development

As indicated in the review of literature, a good correlation between off-flavour development and TBA values in a number of products has been reported by several investigators. The main purpose of this

investigation was to determine the relationship between off-flavour development and TBA numbers in frozen green beans. Each sample was tested and the results are reported in Tables 2 to 19 and in Figures 2 to 19. Organoleptic tests were carried out simultaneously with the TBA test, and flavour scores were assigned them.

Relationship Between Flavour Scores and TBA Numbers

The off-flavour development of beans can be described as the formation and accumulation of malodorous bitter-tasting compound or compounds. In this experiment, four flavour scores were used: good, slightly off, off, very off. The last term was used for a very wide range of flavours ranging from a strong and definitely unpleasant bitterness to an unbearable bitter flavour. The term "off" was used in cases where the off-flavour was definite but still not very strong. "Slightly off" was used in cases where a mild off-flavour was noted.

In Series I it was difficult to establish a satisfactory relationship between flavour scores and TBA numbers due to the errors in method previously explained. However, at the end of the experiment, very pronounced off-flavours were present in the unblanched samples

under oxygen at all temperatures used while in nitrogen, the unblanched samples developed flavours which were "very off," "off," and "slightly off" at temperatures of -9.4°, -15.0° and -20.6°C, respectively. In air, the unblanched samples developed flavours which were "very off," "very off" and "off" at temperatures of -9.4°, -15.0° and -20.6°C, respectively. The TBA numbers for unblanched samples stored in nitrogen were 1.40, 1.26 and 1.04, and for those stored in air, 1.46, 1.34 and 1.16, at temperatures of -9.4°, -15.0° and -20.6°C, respectively. This indicates that the TBA numbers increased as off-flavours developed.

The blanched material in Series I did not develop off-flavours as indicated by the flavour scores. This is in agreement with TBA numbers which remained fairly constant.

In Series II a very definite relationship existed between flavour scores and TBA numbers. For unblanched material stored under oxygen, nitrogen, and air, at all temperatures used, the relationship was: flavour score of "good" up to TBA number of 1.10; flavour score of "slightly off" from TBA numbers of 1.10 to 1.20, flavour score of "off" from TBA numbers of 1.20 to 1.28; and flavour score of "very off" from TBA numbers of 1.28 and up.

In Series III there also appeared to be a very definite relationship between the flavour scores and TBA numbers. Beans stored in oxygen, nitrogen and air at all storage temperatures gave the following results: flavour score of "good" up to TBA number of 1.09, flavour score of "slightly off" from TBA numbers of 1.09 to 1.19, flavour score of "off" from TBA numbers of 1.19 to 1.28, and flavour score of "very off" from TBA numbers of 1.30 and up.

In Series II and III the blanched material did not develop off-flavours and the TBA numbers varied only slightly.

Commercial samples of frozen green beans were tested with the TBA test as outlined. TBA numbers of 0.62 and 0.54 were obtained. These values are lower than those obtained for adequately blanched beans in this investigation and are well within the range of TBA numbers associated with good quality.

The results of this investigation, using the TBA reagent on frozen green beans indicate that there appears to be a satisfactory relationship between off-flavour development and the TBA numbers obtained. The TBA reagent could, therefore, be used to trace the development of off-flavour in frozen green beans.

Effect of Blanching on Off-flavour Development

The strongest off-flavour always developed in raw samples. However, a 3-min blanch was sufficient to inactivate completely the enzymes responsible for off-flavour development.

Effect of Storage Temperature on Off-flavour Development

The strongest off-flavour always developed at the highest temperature in the raw samples, as indicated by the results reported in Tables 2 to 19. As the storage temperature decreased to -15.0°C the development of off-flavour slowed down and by the end of the storage period was not as great as that in the raw material at -9.4°C. At the lowest storage temperature, -20.6°C, the development of off-flavour was very slow and in Series II and III off-flavour did not develop throughout the storage period when stored in nitrogen and air. Furthermore, the most rapid development of off-flavour always occurred in the unblanched samples stored at the highest temperatures.

Effect of Composition of Atmosphere in the Container on Off-flavour Development

The atmosphere in the container played a definite role in off-flavour development of frozen beans during storage in all three series. This is clearly demonstrated when the effect of storage atmosphere at the highest temperature, -9.4°C, is examined, Tables 20 to 22. The effect of the gas treatments at all temperatures upon off-flavour development is recorded in Tables 2 to 19. The development of off-flavour is found to occur most rapidly in the atmospheres used in the following order: oxygen > air > nitrogen.

As conditions go from completely aerobic (oxygen) to completely anaerobic (nitrogen) the development of off-flavour slows down although it still occurs. This would suggest that a different mechanism is involved in the development of off-flavour in frozen beans stored in oxygen and nitrogen. In both cases, however, enzymic activity appears to be involved, since adequate blanching prevents the development of off-flavour and production of TBRS. In nitrogen, off-flavour formation is probably due to anaerobic respiration and the action of hydrolytic enzymes. In oxygen, the action of oxidative enzymes, such as lipoxidase, probably plays a greater role. This

would also hold true in samples stored in air but at a slower rate because of the lower concentration of oxygen.

Characteristics of TBRS

Malonaldehyde was prepared by the acid hydrolysis of 1,1,3,3-TEP according to the method of Kwon and Watts (1963). The ultraviolet absorption spectrum of the malonaldehyde obtained was recorded on a Bausch and Lomb Spectronic 505, after adjusting the pH to 1.6, and is shown in Figure 23. The compound showed a peak at 245 mμ. This is in accordance with the absorption maximum of 245 mμ for malonaldehyde at pH lower than 3.0, reported by Kwon and Watts (1963).

The ultraviolet absorption spectrum of a steam distillate of unblanched beans stored under oxygen for 147 days at -9.4°C was studied and is shown in Figure 24. The steam distillation was carried out at pH 1.5 since Tarladgis et al. (1960) reported that maximum recovery of TBRS from oxidized food by steam distillation was obtained only under acid conditions. This is also in accordance with work done by Kwon and Watts (1963, 1964) and Kwon et al. (1965). The steam distillate was adjusted to pH 1.6 with 3 N HCl. Absorption maxima for the bean distillate were found to occur between 210 and

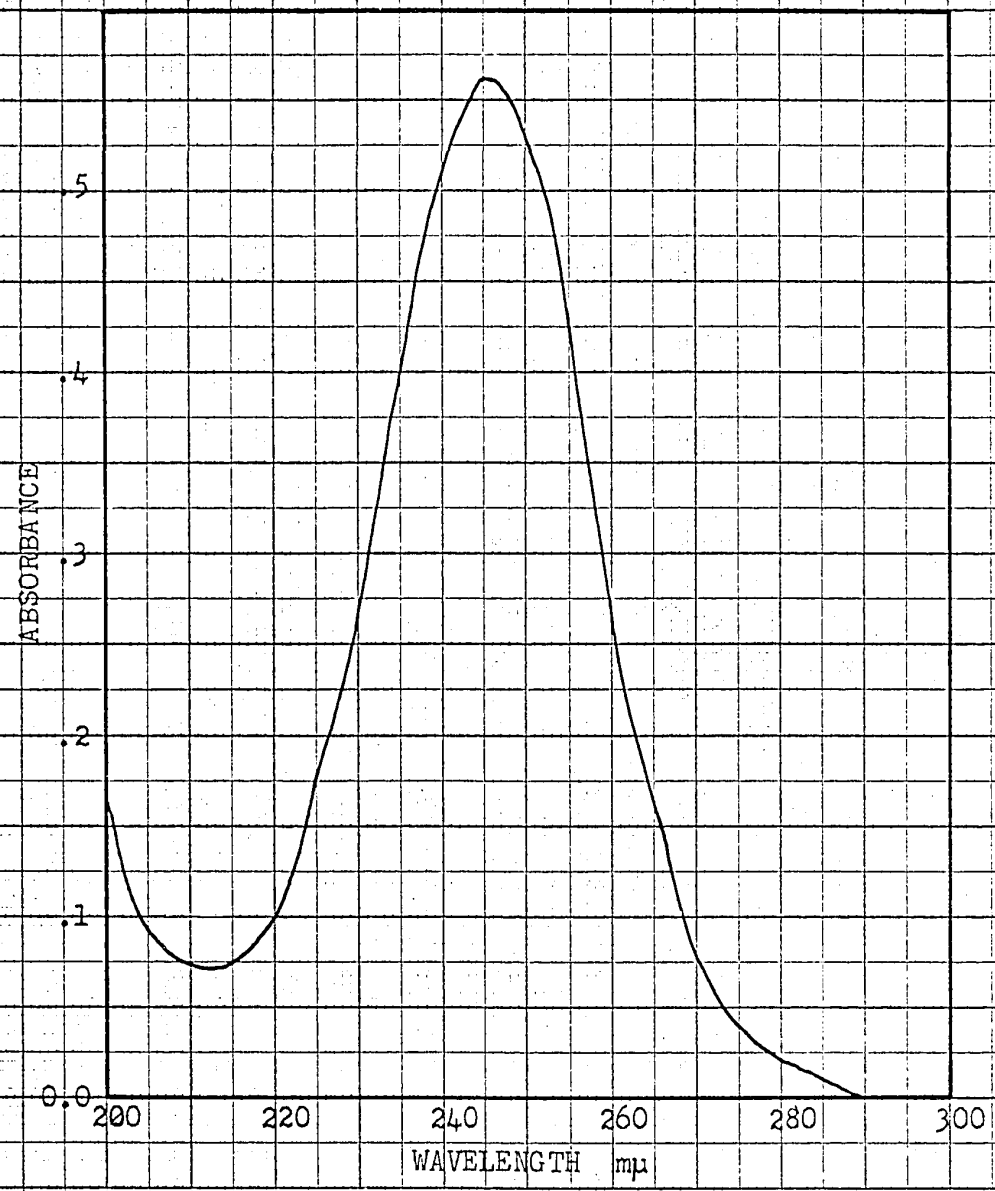

Fig. 23.--Ultraviolet absorption spectrum of a sample of MA obtained from the acid hydrolysis of 1,1,3,3-TEP. The pH of the MA adjusted to 1.6.

Fig. 24.--Ultraviolet absorption spectrum of a steam distillate obtained from a sample of acidified (pH 1.1) unblanched beans stored under oxygen for 147 days at -9.4°C. The pH of the distillate adjusted to 1.6.

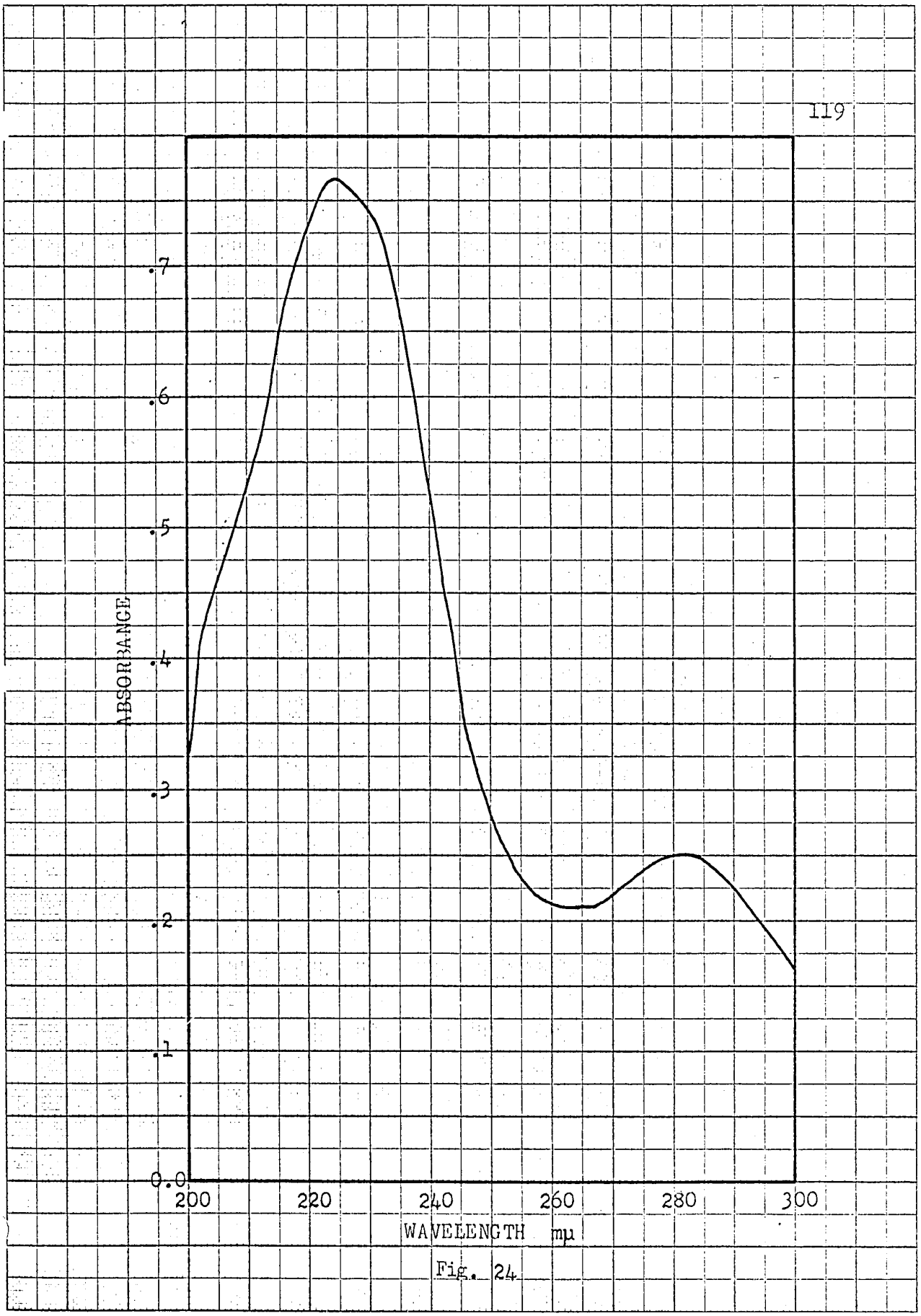

Fig. 24

225 mμ and between 278 and 288 mμ. Kwon and Watts (1963) reported that the saturated aldehydes, formaldehyde, acetaldehyde, propionaldehyde, butyraldehyde, and hexaldehyde show absorption peaks in the range of 278 to 288 mμ. They also reported that the α,β-unsaturated aldehydes, acrolein and crotonaldehyde, absorb in the region of 210 to 225 mμ. These absorption maxima are not affected by the low pH employed.

The absorption spectrum of the coloured complex produced with the TBA reagent and MA obtained by the method of Kwon and Watts (1963) is shown in Figure 25. The TBA-MA complex had an absorption maximum of 532 mμ. The absorption spectrum of the coloured complex produced by the reaction of TBA and TBRS from an alcohol extraction of beans had an absorption maximum of 535 mμ.

A comparison of the 2,4-DNPHs of the MA and of the steam distillate of the unblanched beans stored in oxygen on thin-layer chromatography was also carried out. Chloroform-methanol (22:1)(V/V) and 95% ethanol were used as solvents. However, in both cases the R_f values were different.

Thus a comparison of Figures 23 and 24 would indicate that MA is not present in frozen stored beans. This is supported by the results obtained with thin-layer

Fig. 25.--I. Absorption spectrum of the coloured complex produced with TBA reagent and MA obtained from the acid hydrolysis of 1,1,3,3-TEP. II. Absorption spectrum of the coloured complex produced with TBA reagent and TBRS obtained from the alcohol extraction of unblanched beans stored under oxygen for 147 days at -9.4°C.

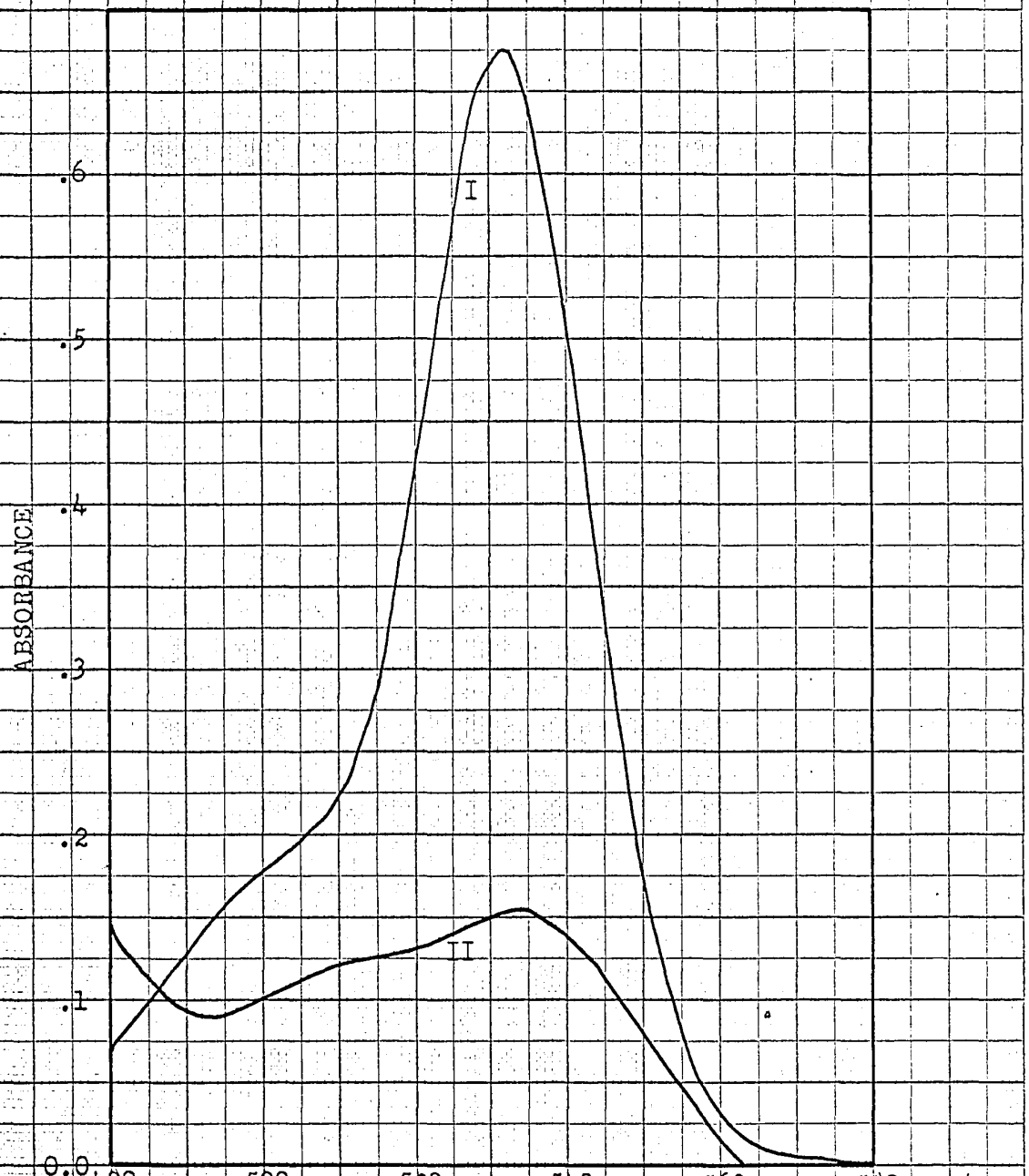

Fig. 25

chromatography. However, the TBA-TBRS complex is quite similar to the TBA-MA complex and would seem to indicate that the compound or compounds reacting with TBA resemble MA.

Identification of 2,4-DNPHs

In Series I an attempt was made to identify the 2,4-DNPHs of the carbonyl compounds in the ethanol extract of unblanched and blanched beans by the use of thin-layer chromatography.

The 2,4-DNPHs were separated by TLC, as previously outlined, until the individual compounds were separated. They were then collected and the absorption spectra were determined from 200 to 800 mμ, on a Bausch and Lomb Spectronic 505. The absorption spectra were compared with the spectra of 2,4-DNPHine derivatives of known carbonyl compounds. A total of 49 individual spots was collected and their absorption spectra determined. However, in the majority of cases, due to the minute amount of material collected it was not possible to compare the spectra of the unknown derivatives with those prepared from known compounds. Satisfactory peaks were not obtained in the visible range where the peaks of the 2,4-DNPHine derivatives of known carbonyl compounds are

found, i.e., in the region of 350-385 mμ.

Absorption spectra were obtained for the most dominant 2,4-DNPHs from blanched and unblanched beans. A comparison of the absorption spectra of a compound found in both blanched and unblanched beans, stored under oxygen at -9.4°C, is shown in Figure 26. A major peak at 444 mμ was obtained for both derivatives. The unidentified compound thus appears to be present in blanched and unblanched beans after extended storage under oxygen at -9.4°C. This compound was also shown to be a bis-aldehyde since it turned blue in the presence of NaOH. Since the blanched beans had still retained good flavour and since the unidentified compound appeared to be one of the ones present in highest concentration it is assumed it did not contribute too greatly to off-flavour development. However, it was present in greater concentration in unblanched beans. The compound would also appear to be quite complex in its make-up as evidenced by the shifting of the 2,4-DNPHine peak so far to the right. As the number of double bonds increases in a compound, the normal 2,4-DNPHine peak of 350 mμ is shifted to the right.

All of the absorption spectra obtained for the unknown derivatives showed strong absorption peaks in

Fig. 26.--Absorption spectra of 2,4-DNPHine derivatives of a compound found in the alcohol extract of beans. I. Unblanched beans stored under oxygen at -9.4°C for 76 days. II. Blanched beans stored under oxygen at -9.4°C for 76 days.

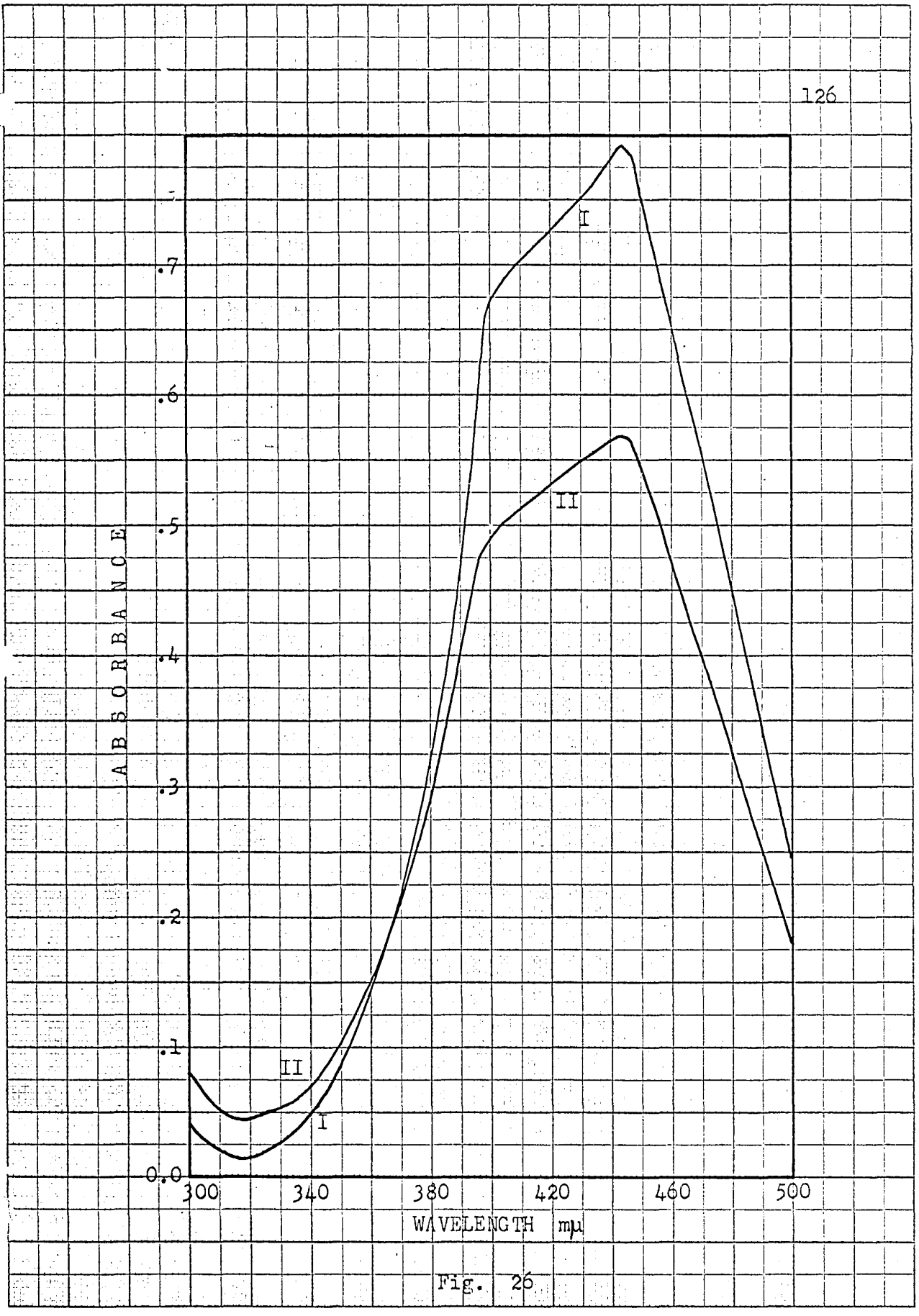

Fig. 26

the region of 210 mμ and/or 225 mμ. Further investigation revealed that when Silica gel G was removed from the plates, after a two-dimensional run with the solvents and treated the same way as the spots collected, an absorption spectrum was obtained with peaks at 212 mμ and 225 mμ. This would indicate that the peaks found for the unknown carbonyl compounds in that region have no significance.

Since it was not possible to identify any of the 2,4-DNPHine derivatives of compounds present in unblanched and/or blanched beans in Series I, efforts to identify the carbonyl compounds of beans were repeated. The 2,4-DNPHine derivatives of the steam distillate of unblanched beans stored under oxygen at -9.4°C for 147 days were used, along with the ethanol extracts of similar stored beans. They were compared with the derivatives of known carbonyl compounds, using TLC, as previously outlined. The unknowns and the standards were spotted on the same plates and developed and the R_f values compared. The results obtained could not confirm the presence of the following compounds in stored frozen beans: acetaldehyde, acrolein, anisaldehyde, benzaldehyde, butyraldehyde, crotonaldehyde, diacetyl, formaldehyde, glyoxal, hexanal, n-hexaldehyde,

malonaldehyde, propionaldehyde, and valeraldehyde.

The 2,4-DNPHine derivative of 1-heptanal appeared to resemble closely one of the spots of the 2,4-DNPHs of the steam distillate, in terms of R_f value and colour appearance. However, when an absorption spectrum of the unknown was compared with that of the 1-heptanal derivative, it was apparent that the two were not the same.

The absorption spectrum of the most predominant 2,4-DNPHine derivative present in the steam distillate of unblanched beans is shown in Figure 27. The absorption maximum is at 374 mμ. Two sub-peaks, which are not shown, were observed at 280 mμ and 254 mμ. Although this compound exhibited absorption in the area expected for 2,4-DNPHs efforts to identify it were unsuccessful.

While the attempts to identify the carbonyl compounds in beans were unsuccessful, it was quite apparent that bis-aldehydes were present since a large number of spots changed to a blue colour upon spraying the chromatograms with a 1% alcoholic NaOH solution.

Fig. 27.--Absorption spectrum of a 2,4-DNPHine derivative of a compound found in the steam distillate of unblanched beans stored under oxygen at -9.4°C for 147 days.

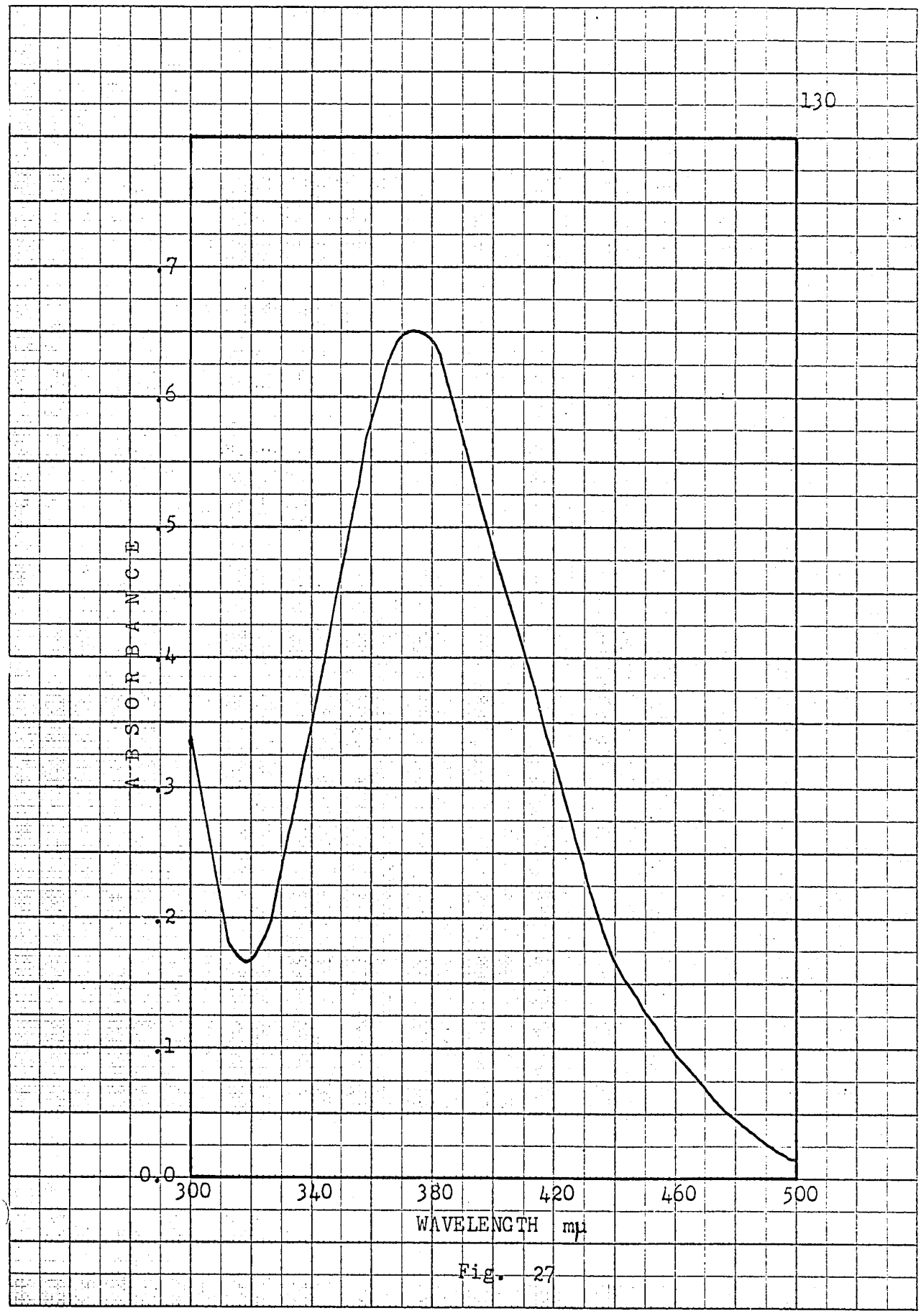

Fig. 27

Steam Distillation vs Ethanol Extraction

A comparison of the steam distillation method and the ethanol extraction method to obtain the TBRS was carried out on both blanched and unblanched beans stored under oxygen at -9.4°C.

Part of the material extracted by each method was reacted with TBA and the absorption spectra were determined with the Bausch and Lomb Spectronic 505. Figure 28 represents a comparison of the coloured complexes produced by the TBA-TBRS reaction for the material extracted by each of the methods. It is quite apparent that the ethanol method extracted much more TBRS from the beans than did steam distillation. The ethanol extract TBA-TBRS complex exhibited absorption maxima at 328, 380, 418, 455 and 535 mμ, while the steam distillate TBA-TBRS complex exhibited absorption maxima at 327, 455 and 535 mμ.

As would be expected since the ethanol extract contained more TBRS from the beans it also contained a greater quantity of compounds reacting with 2,4-DNPHine. TLC of the 2,4-DNPHs obtained from the steam distillates and the alcohol extracts revealed that many more hydrazones were formed in the alcohol extract. This would be expected since only the volatile carbonyl compounds are

Fig. 28.--A comparison of the absorption spectra of the TBA-TBRS coloured complexes obtained from beans. I. Alcohol extract of unblanched beans stored under oxygen for 20 days at -9.4°C.
II. Steam distillate of unblanched beans stored under oxygen for 20 days at -9.4°C.

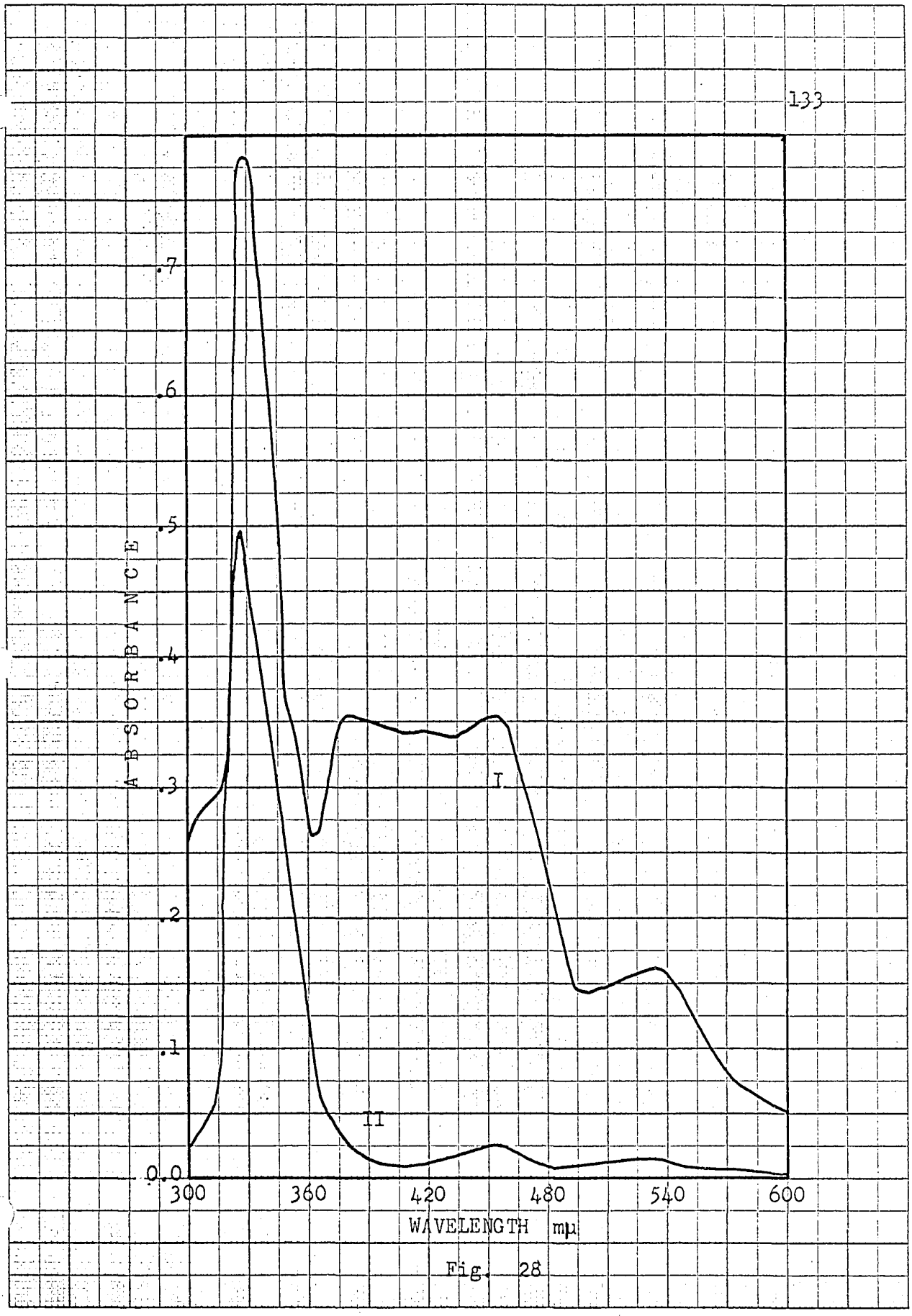

Fig. 28

obtained by steam distillation. However, in the alcohol extract a wider range of compounds would be extracted and form 2,4-DNPHs. Photographs were taken of the developed chromatograms of the 2,4-DNPHs of the unblanched and blanched bean extracts throughout the storage period.

Figures 29 and 30 show that, initially, the unblanched and blanched beans contain an approximately equal quantity of reactive material.

After 13 days of storage at -9.4°C, photographs of the 2,4-DNPHs were again taken and are shown in Figures 31 and 32. They indicate that there was more reactive material in the unblanched samples than in the blanched. The spots located in the top left and bottom right sides of the plate show the greatest increase in intensity for the unblanched beans. No increase was observed for the blanched beans.

After 20 days of storage at -9.4°C, photographs of the 2,4-DNPHs were again taken and are shown in Figures 33 and 34. They indicate that the quantity of reactive material in unblanched beans had continued to increase, as indicated by the increased colour intensity of the spots situated in top left and bottom right sides of the plate. However, the quantity of reactive material in blanched beans did not appear to increase.

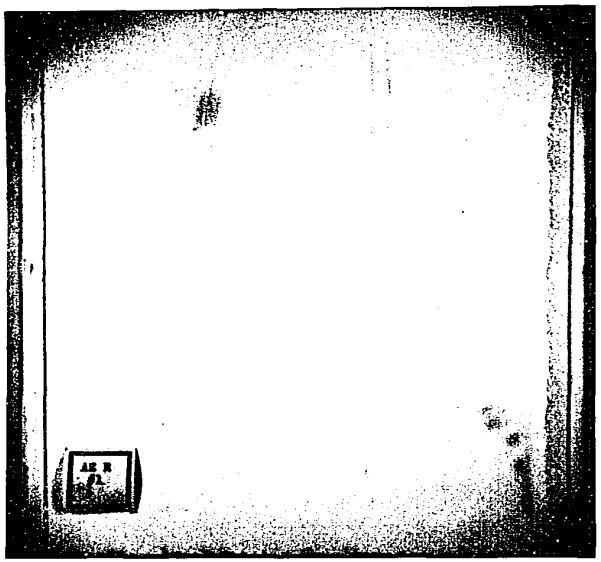

Fig. 29.--Chromatogram of 2,4-DNPHs obtained from the ethanol extract of fresh unblanched beans.

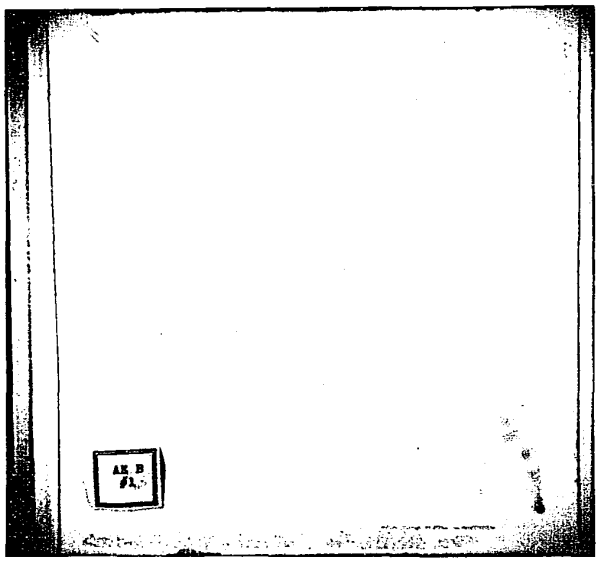

Fig. 30.--Chromatogram of 2,4-DNPHs obtained from the ethanol extract of fresh blanched beans.

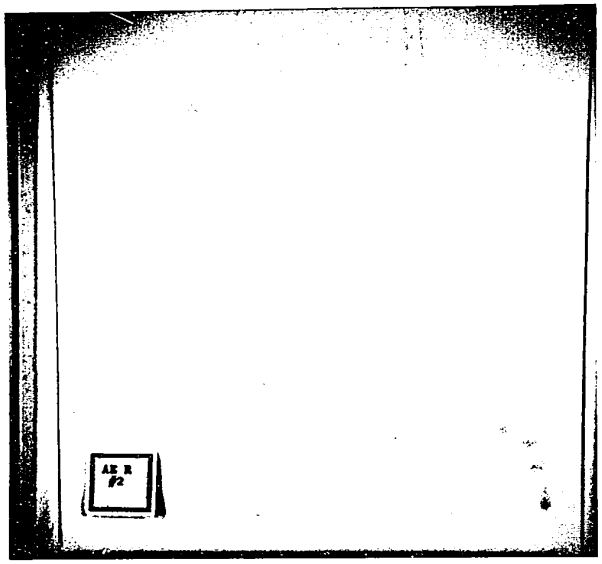

Fig. 31.--Chromatogram of 2,4-DNPHs obtained from the ethanol extract of unblanched beans stored under oxygen at -9.4°C for 13 days.

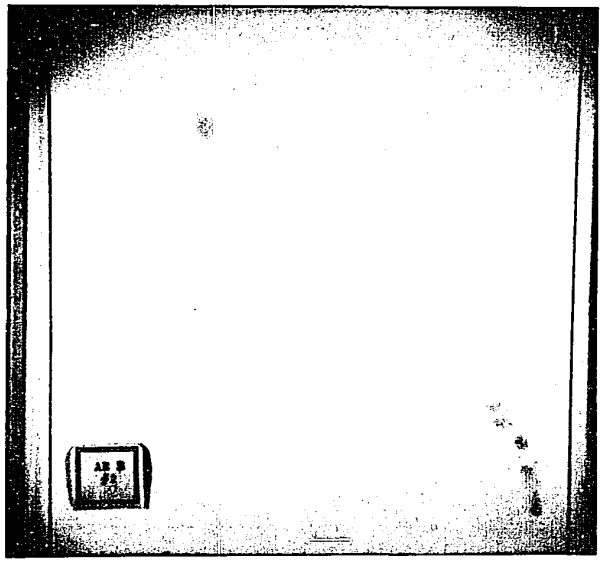

Fig. 32.--Chromatogram of 2,4-DNPHs obtained from the ethanol extract of blanched beans stored under oxygen at -9.4°C for 13 days.

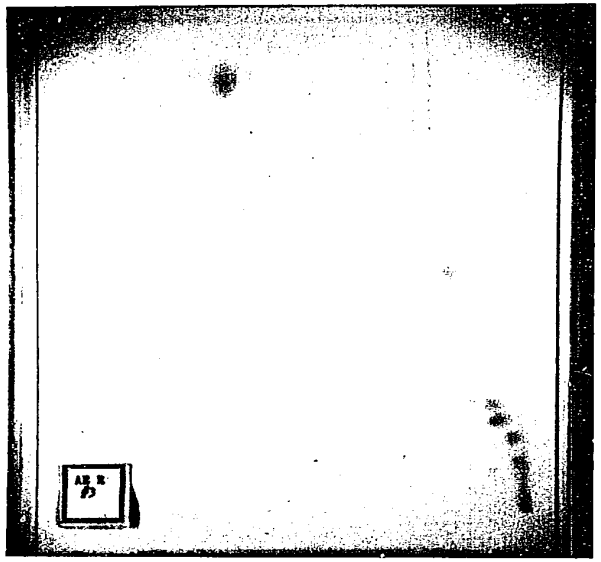

Fig. 33.--Chromatogram of 2,4-DNPHs obtained from the ethanol extract of unblanched beans stored under oxygen at -9.4°C for 20 days.

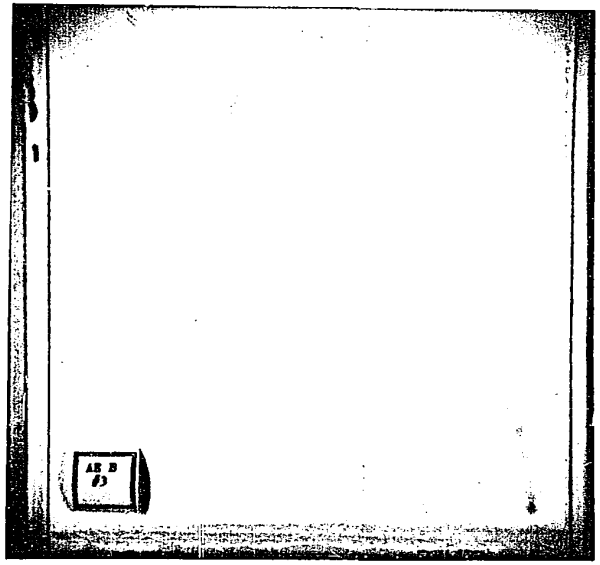

Fig. 34.--Chromatogram of 2,4-DNPHs obtained from the ethanol extract of blanched beans stored under oxygen at -9.4°C for 20 days.

These findings parallel those obtained in the TBA test. The TBA numbers obtained initially, at 13 days and at 20 days were 0.740, 1.42 and 1.45, respectively, for unblanched beans and .587, .545 and .580, respectively, for blanched beans, stored in oxygen at -9.4°C. Thus as the TBA number increased in unblanched beans so did the intensity of some of the 2,4-DNPHs on the TLC plates. Furthermore, the TBA number for blanched beans did not change appreciably and increase in intensity was not observed on the chromatograms of blanched beans.

Colour photographs of the developed chromatograms of the unblanched and blanched beans were taken (Figures 35 and 36) in order to have a permanent record of the appearance of the spots obtained.

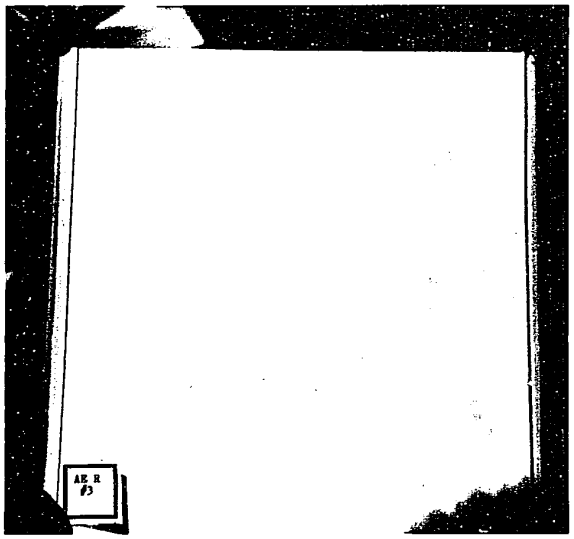

Fig. 35.--Chromatogram of 2,4-DNPHs obtained from the ethanol extract of unblanched beans stored under oxygen at-9.4°C for 20 days.

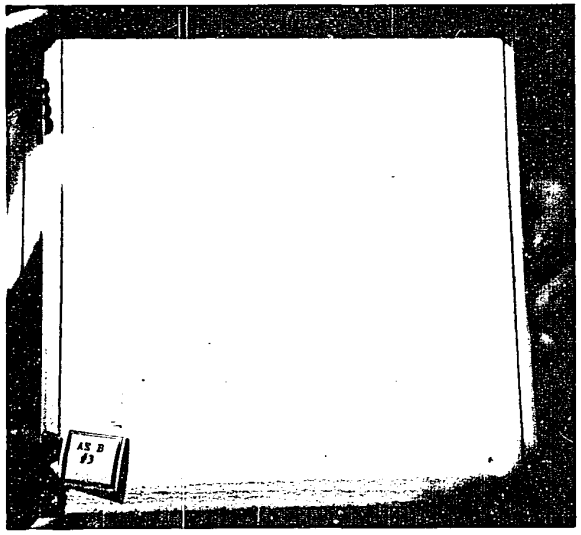

Fig. 36.--Chromatogram of 2,4-DNPHs obtained from the ethanol extract of blanched beans stored under oxygen at -9.4°C for 20 days.

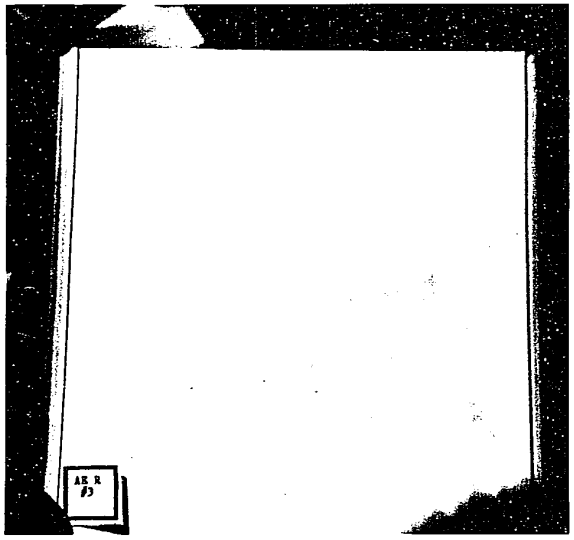

Fig. 35.--Chromatogram of 2,4-DNPHs obtained from the ethanol extract of unblanched beans stored under oxygen at-9.4°C for 20 days.

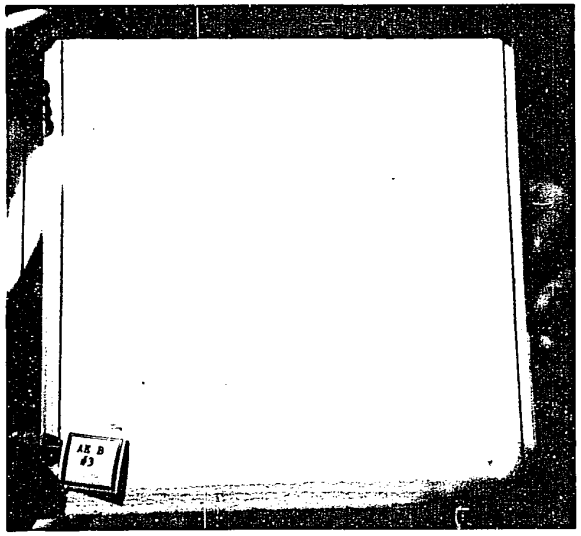

Fig. 36.--Chromatogram of 2,4-DNPHs obtained from the ethanol extract of blanched beans stored under oxygen at -9.4°C for 20 days.

IV. SUMMARY AND CONCLUSIONS

Unblanched and blanched beans were stored under oxygen, nitrogen and air at -9.4°C, -15.0°C and -20.6°C. Three different lots were investigated during 76, 42 and 42 days respectively. The development of oxidative rancidity was investigated, using 2-TBA as the reagent. Organoleptic tests were carried out concurrently.

The 2-thiobarbituric acid reactive substance (TBRS) was satisfactorily extracted from the beans, using 95% ethanol as the solvent. Ethanol extracts of the unblanched and blanched beans, stored under the above conditions, were analyzed at regular intervals. The results indicate that the TBA method is sensitive enough to detect changes occurring in raw beans, and that these changes parallelled the development of off-flavour in the beans, as determined organoleptically. A satisfactory relationship appears to exist between the development of off-flavours and TBA numbers in frozen green beans. Thus the TBA reagent could be used to detect the development of off-flavour in frozen green beans.

The accumulation of TBRS and the development of

off-flavour were found to be influenced by handling methods and storage conditions. Blanching was found to prevent both TBRS production and off-flavour development. In unblanched beans the storage atmosphere and temperature used had a definite effect on TBRS production and off-flavour development, and this effect was exerted in the following order: oxygen > air > nitrogen and $-9.4^{o}C > -15.0^{o}C > -20.6^{o}C$.

The presence of carbonyl compounds in the beans was demonstrated by preparing their 2,4-dinitrophenylhydrazine derivatives and examining them by means of thin-layer chromatography.

A comparison of the steam distillation method of extraction and the ethanol extraction method was carried out. Results indicate that the ethanol extract of beans contains more TBRS and more carbonyl compounds than does the steam distillate of beans.

Malonaldehyde could not be identified in the blanched or unblanched beans after extended periods of storage. Thus it is assumed that the 2-TBA reacts with some other compound or compounds to form the coloured complex obtained.

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