1939

# Effects of tomato juice on production of flavor contributants in butter cultures

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# NOTE TO USERS

# EFFECTS OF TOMATO JUICE ON PRODUCTION OF FLAVOR CONTRIBUTANTS IN BUTTER CULTURES

by

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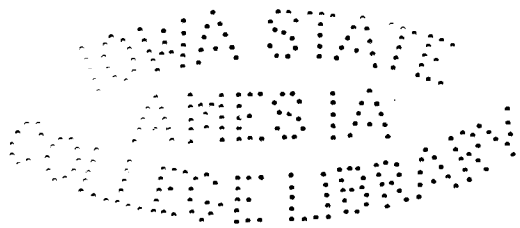
A Thesis Submitted to the Graduate Faculty
for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject Dairy Bacteriology

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1939

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# INTRODUCTION

Fine butter made from cream to which butter culture has been added has a flavor and aroma due to the formation, by bacterial action, of materials not present in the milk at the time of secretion (33). Two groups of substances (15, 11, 23) have been recognized as contributing to the flavor. Volatile acids, including acetic and traces of propionic, form the one group. The other consists of neutral substances, called by van Beynum and Pette (35) the $C_4$ compounds. This group includes diacetyl ($CH_3.CO.CO.CH_3$), acetylmethylcarbinol ($CH_3.CO.CHOH.CH_3$) and 2,3-butylene glycol ($CH_3.CHOH.CHOH.CH_3$). Of these, diacetyl is the only one possessing an odor (21), but the relationship between all three is so close that they are usually considered together. Diacetyl is the only diketone which has been detected in butter and butter cultures in significant amounts (14).

Diacetyl has been shown to be of considerable, if not primary, importance in the production of flavor in butter, and therefore any method whereby the production of diacetyl and acetylmethylcarbinol, which is claimed to be its precursor, in a culture can be increased will be of great value.

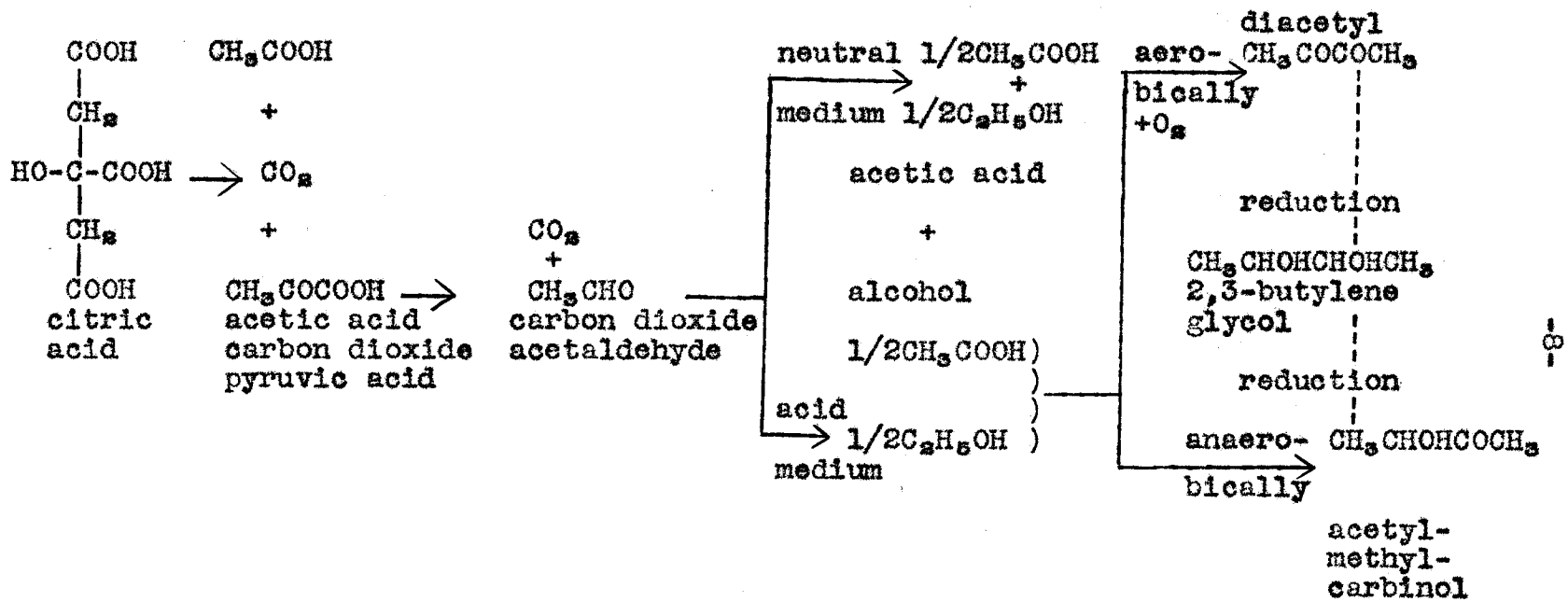
# HISTORICAL

When the citric acid fermenting streptococci, Streptococcus citrovorus and Streptococcus paracitrovorus, were first isolated (10), butter cultures were prepared by obtaining a number of strains of each organism and mixing them with various strains of Streptococcus lactis until a satisfactory culture was developed (7). The activity and suitability of the cultures produced in this way were determined by studying the flavor, aroma and volatile acidity (6) of the culture after incubation. It was found that the production of volatile acid depended on the total acidity (5). A satisfactory pH could be obtained by growing the citric acid fermenting organisms in association with Streptococcus lactis or by acidifying the medium with organic or mineral acids. When citric acid was used, the volatile acid production was greatly increased because this acid is the substrate which is fermented to yield volatile acids (9).

Further studies revealed that diacetyl also plays an important part in the flavor and aroma of butter cultures (38). When citric acid is fermented various neutral products are formed in addition to the volatile acids. According to some investigators (27), the main neutral product formed by the organisms is acetylmethylcarbinol; in neutral medium (16) this is reduced to 2,3-butylene glycol while in acid medium, under

aerobic conditions, it is partially oxidized to diacetyl (27). This oxidation reaction, however, never reaches completion, and a butter culture regularly contains much more acetylmethylcarbinol than diacetyl (21).

van Beynum and Pette (35) do not share this view and they consider that acetaldehyde is formed by the organisms and that this substance (36) is the common precursor of all three neutral compounds. They believe that in a neutral medium two molecules of acetaldehyde undergo a Cannizaro reaction to form one molecule of acetic acid and one of ethyl alcohol. In an acid medium there are two possible courses which the fermentation may follow. If conditions are anaerobic the acetic acid and alcohol, formed from the acetaldehyde, combine to form one molecule of acetylmethylcarbinol; under aerobic conditions, on the other hand, they combine to form diacetyl. Both these compounds may subsequently be reduced to 2,3-butylene glycol. Acetylmethylcarbinol is always present in excess because only at the surfaces of the cultures are conditions sufficiently aerobic for the formation of diacetyl. The scheme of van Beynum and Pette (35) for the decomposition of citric acid is given below:


COOH
CH2
HO-C-COOH
CH2
COOH
citric acid
CH3COOH
+
CO2
+
CH3COCOOH
acetic acid
carbon dioxide
pyruvic acid
CO2
+
CH3CHO
carbon dioxide
acetaldehyde
neutral 1/2CH3COOH
+
medium 1/2C2H5OH
acetic acid
+
alcohol
1/2CH3COOH)
)
acid )
1/2C2H5OH )
medium
aero-
bically
+O2
diacetyl
CH3COCOCH3
reduction
CH3CHOHCHOHCH3
2,3-butylene
glycol
reduction
anaero-
bically
CH3CHOHCOCH3
acetyl-
methyl-
carbinol

When the importance of diacetyl as a flavor contributant was realized, many attempts were made to increase the yield obtained from butter cultures. One of the earliest methods (7) was to isolate numerous strains of Streptococcus lactis and one or both citric acid fermenting organisms, Streptococcus paracitrovorus, and then make various combinations of Streptococcus lactis and the latter organisms until one was obtained which had the desirable qualities of a good butter culture. Later, substances which might be fermented by butter culture organisms with the production of acetylmethylcarbinol plus diacetyl were sought (24) and it was found that citric acid was a substrate which was very readily attacked. The effect of pH (26) was also studied and various acids were used to lower it to the required level. While satisfactory results could be obtained with mineral and certain organic acids, the most striking results were obtained when citric acid was used, as it not only adjusted the pH but also provided an abundance of a suitable substrate (24).

Butter cultures were incubated at various temperatures (8), and it was found that at relatively high temperatures there was a production of various off flavors while at temperatures significantly below 21° C. bacterial activity was slowed to such an extent that maximum production of flavor materials did not occur and the cultures were lacking in flavor. The effect of holding cultures for various periods

was also studied (19) and it was noted that the most satisfactory results were obtained when the cultures were allowed to develop a suitable pH at 21° C. and then held at a low temperature until 48 hours after inoculation. Under these conditions reduction of diacetyl to 2,3-butylene glycol did not occur as readily as at higher temperatures, and diacetyl tended to accumulate. Attempts to prevent reduction of diacetyl by adding oxidizing agents to cultures were not successful (18), but it was found that when cultures were grown in a tank and saturated with oxygen or aerated with air under pressure a considerable increase in yield of diacetyl was obtained (3). An attempt was made to increase the production of acetylmethylcarbinol plus diacetyl by adding various substances of both simple and complex chemical nature to the milk in which the cultures were grown (1). These were not intended to serve as substrates but rather to increase the activity of the organisms. Substances used were grouped as energy sources, nitrogen sources, products of bacterial activity and accessory factors. Certain of the accessory factors, iso-ascorbic acid, beef infusion and tomato juice, caused very marked increases in yields of acetylmethylcarbinol plus diacetyl. Various percentages of tomato juice were added to butter cultures and the yields of acetylmethylcarbinol plus diacetyl compared with those from cultures containing no

tomato juice. Typical results from such a series of determinations are given below:

| Amount of juice in milk inoculated with butter culture after adding 0.15 % citric acid | Net increase in yield of acetylmethylcarbinol plus diacetyl in culture containing juice | Per cent increase in yield of acetylmethylcarbinol plus diacetyl |
|---|---|---|
| | Mg. Ni-salt per 200g. | |
| 1 % | 6.6 | 31.1 |
| 3 % | 12.8 | 63.2 |
| 5 % | 21.0 | 94.5 |
| 10 % | 29.3 | 114.1 |
| 15 % | 32.4 | 74.6 |
| 20 % | 25.6 | 92.2 |
| 30 % | 11.1 | 12.2 |
| 40 % | -20.8 | -41.2 |

## PURPOSES OF THE STUDY

Since tomato juice has such a marked effect on the production of acetylmethylcarbinol plus diacetyl in butter cultures, its action was studied in greater detail. Both fresh and canned tomatoes were used in the preliminary trials (1) and there seemed to be an indication that some brands of canned tomatoes were not as effective as others in increasing the yield. To determine whether such differences actually existed the effects of juices from a number of brands of commercially canned tomatoes were compared with those of juices from fresh tomatoes.

An attempt was made to correlate certain characteristics of the different brands of juice with their effects on the yield of acetylmethylcarbinol plus diacetyl.

The amounts of acetylmethylcarbinol plus diacetyl present at various stages of the fermentation were studied in order to determine whether tomato juice stimulated or accelerated the production of these compounds. The action would be regarded as stimulatory if the maximum amount of acetylmethylcarbinol plus diacetyl were increased by the addition of tomato juice and as acceleratory if the maximum content were essentially the same in the cultures with and without juice, but the rate of production were increased. The suggestion has been made (12) that the mechanism of the breakdown of citric acid may not be the same with all strains of citric acid fer-

menting streptococci. If this be the case, it is entirely possible that the response to tomato juice might vary with the strain of organism present in the butter culture. Accordingly, a study was made of the effect of tomato juice on the production of acetylmethylcarbinol plus diacetyl by a number of butter cultures.

Tomato juice is definitely acid and to determine whether its effect on the production of acetylmethylcarbinol plus diacetyl is in any way related to its acidity, samples of juices were neutralized and the effects of these compared with those of untreated juices.

To determine whether Streptococcus lactis or the citric acid fermenting organisms were affected most by tomato juice, these organisms were isolated and the action of the juice on the production of acid by Streptococcus lactis and on the production of acetylmethylcarbinol plus diacetyl by the citric acid fermenting streptococci was noted.

A method was sought that would yield a concentrated, highly potent and stable material from tomatoes, which could readily be submitted to analytical procedures.

A study was made of various factors which might modify the effect of tomato juice on the yield of acetylmethylcarbinol plus diacetyl by butter cultures in order to develop a method of preparing the juice in which its potency would

not be reduced.

Preliminary studies were made to find the fraction of tomato juice responsible for its effect on the production of acetylmethylcarbinol plus diacetyl.

# METHODS

## I. Cultural

Throughout the studies whole milk was used as the culture medium for the organisms. It was pasteurized by heating in a flask, which was partially submerged in boiling water for 1 hour and then cooled as rapidly as possible in running water.

Citric acid was added at the rate of 0.15 per cent in the form of a 60 per cent aqueous solution. The pasteurized and cooled milk was agitated vigorously and the citric acid added slowly in order to avoid excessive coagulation. It was then inoculated with 2 to 3 per cent of butter culture.

The butter cultures used were those maintained by the Dairy Industry Section of the Iowa Agricultural Experiment Station. Most of them are regularly employed as mother cultures for the preparation of commercial lots of butter culture and these seem to give satisfactory results in the factories to which they are sent.

Unless otherwise stated, 300 ml. portions of the inoculated milk were used in the trials. The portions were measured into sterile, cotton stoppered, pint milk bottles with a sterile graduated cylinder. When liquids were added to the cultures they were measured into the bottles first and then enough milk was added to make the total volume in each bottle 300 ml.

In some of the experiments the controls were duplicated, but the cultures containing the test materials were not, as it was found that duplicates seldom differed significantly and it was felt that more information could be gained by repeating the whole trial. The cultures were incubated in a constant temperature incubator at 21° C. for 15 to 18 hours unless otherwise stated.

## II. Preparation of Tomato Juices

Most of the tomato juice used was prepared from commercially canned tomatoes, although juice from fully ripened fresh tomatoes was occasionally employed for comparative purposes. The canned tomatoes were strained through a double layer of cheesecloth. The first juice to come through contained considerable solid matter but this was returned to the filter and in a short time a clear juice was obtained. The juice was then dispensed into screw-capped 6 ounce bottles, 100 ml. to a bottle, and sterilized at 15 pounds pressure for 25 minutes. The fresh tomatoes used were dipped into boiling water, peeled, sliced and forced through a vegetable masher. The pulp obtained in this manner was then filtered and sterilized in the same way as the juice from canned tomatoes.

## III. Analytical

### A. Determination of acetylmethylcarbinol plus diacetyl.

The acetylmethylcarbinol plus diacetyl value was determined by the Lemoigne-van Niel method (37) as applied by Michaelian, Farmer and Hammer (22). A 200g. portion of the ripened culture was steam distilled after adding 40 ml. of a 40 per cent ferric chloride solution to oxidize the acetylmethylcarbinol to diacetyl.

Between 100 and 120 ml. of distillate were collected in 10 ml. of a mixture of hydroxylamine hydrochloride (20 per cent solution) 1 part and sodium acetate solution (20 per cent solution) 2 parts. The tip of the adapter was placed below the surface of the mixture so that the volatile diacetyl would not escape. The distillate was heated to about 85° C. and 10 ml. of a 10 per cent solution of nickel chloride were added to precipitate nickel dimethylglyoximate. After the addition of nickel chloride the mixture was allowed to stand for at least 3 days at room temperature to ensure as complete precipitation as possible of the nickel salt. After this, the nickel salt was washed into a weighed sintered glass crucible (Schott-Jena), dried at 110° C., allowed to cool in a desiccator and the weight of the precipitate obtained. In this procedure the diacetyl produced in the culture and that formed by the oxidation of the acetylmethylcarbinol is distilled into

the hydroxylamine hydrochloride-sodium acetate solution where it forms dimethylglyoxime. When nickel chloride is added, a red precipitate of almost insoluble nickel dimethylglyoximate is formed.

All the results are expressed as milligrams of nickel salt equivalent to acetylmethylcarbinol plus diacetyl per 200g. culture. About 84 per cent of the acetylmethylcarbinol present in the culture is recovered by this procedure (32).

B. Determination of pH.

All pH determinations were made electrometrically using a quinhydrone electrode and a saturated calomel half cell.

# EXPERIMENTAL

## Part I

### Comparison of Effects of Juices from Various Brands of Canned Tomatoes and from Fresh Tomatoes on Yield of Acetylmethylcarbinol plus Diacetyl by a Butter Culture

It seemed possible that juices from various brands of canned whole tomatoes, and canned tomato juices might differ in their action on butter cultures, because of differences in composition or in the canning processes used.

To determine whether such differences existed, samples of four brands of canned tomatoes processed in the United States were obtained. In the trials which follow these were designated A, B, C and D. A brand of canned tomatoes grown and packed in the Union of South Africa was designated J, and three brands of American canned tomato juice were designated E, F and I. As a basis of comparison, juice was prepared from fresh, ripe, red tomatoes and from fresh, ripe, yellow tomatoes grown at Ames, Iowa.

Each type of juice was added, in two concentrations — 5 per cent and 10 per cent —, to a butter culture containing added citric acid, and the culture was incubated 14 to 18 hours at 21° C. To reduce the possibilities of variation in results, due to causes other than differences between the lots of juice, the same butter culture,F, was used in all determinations. In a

further attempt to reduce experimental error, the trials were repeated four times. The means of these four trials were determined and these means are the data reported in Table I.

The "controls" are those cultures to which no tomato juice was added. It will be noted that the mean values of the controls are not all the same. Only a limited number of determinations could be made at one time, and, as it required six runs to obtain the necessary data, there are six different values for the controls. The "net difference" was obtained by subtracting the mean value of the controls from the mean value of the trials with tomato juice. The "per cent increase" is the net difference expressed as a percentage of the corresponding control value.

When 5 per cent of juice was added to the culture, the fresh, red and yellow tomatoes and brands A and B caused the largest increases in yield, and no significant* differences could be observed in the effects of the juices from these four sources. Brand C was significantly lower in activity than brands A and B but was still very active. Brands D and J caused only slight increases,while the remaining brands caused significant decreases, all of which were about the

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*Earlier work (2) indicated that differences of 6 mg. are definitely significant when the yields of acetylmethylcarbinol plus diacetyl are between 30 and 100 mg. of Ni-salt per 200g. of culture.

TABLE I

EFFECTS OF JUICES FROM VARIOUS BRANDS OF CANNED TOMATOES AND FROM FRESH TOMATOES ON YIELD OF ACETYLMETHYLCARBINOL PLUS DIACETYL BY A BUTTER CULTURE

Cultures incubated 14-18 hours.

| Type of juice | 5 per cent juice added | | | | 10 per cent juice added | | | |
|---|---|---|---|---|---|---|---|---|
| | Mg. Ni-salt* | | | Per cent increase in yield of Ni-salt* | Mg. Ni-salt* | | | Per cent increase in yield of Ni-salt* |
| | Mean of control trials | Mean of trials with juice | Net difference | | Mean of control trials | Mean of trials with juice | Net difference | |
| Juice from red tomatoes | 63 | 88 | +25 | 39.7 | 61 | 87 | +26 | 42.5 |
| Juice from yellow tomatoes | 63 | 87 | +24 | 39.0 | 61 | 82 | +21 | 34.4 |
| Brand A | 61 | 84 | +23 | 37.7 | 61 | 77 | +16 | 26.2 |
| Brand B | 59 | 82 | +23 | 39.0 | 61 | 70 | + 9 | 14.7 |
| Brand C | 61 | 80 | +19 | 31.2 | 61 | 77 | +16 | 26.2 |
| Brand D | 60 | 68 | + 8 | 13.3 | 63 | 63 | 0 | 0 |
| Brand E | 65 | 58 | - 7 | -10.8 | 65 | 55 | -10 | -15.4 |
| Brand F | 50 | 44 | - 6 | -12.0 | 65 | 55 | -10 | -15.4 |
| Brand H | 65 | 60 | - 5 | - 7.7 | 65 | 55 | -10 | -15.4 |
| Brand I | 65 | 58 | - 7 | -10.8 | 65 | 57 | - 8 | -12.3 |
| Brand J | 47 | 52 | + 5 | 10.0 | 47 | 50 | + 3 | + 6.4 |

*Mg. Ni-salt equivalent to acetylmethylcarbinol plus diacetyl per 200g. culture.

same in magnitude.

When 10 per cent of juice was added to the cultures, there was greater variation in the results obtained. The juices from fresh, red and yellow tomatoes were most effective but the addition of 10 per cent of them did not give greater increases than the use of 5 per cent. Brand A was still active, but did not bring about as large an increase as it did in the lower concentration. The effectiveness of Brand B was greatly reduced but it still produced a significant increase. Brand C appeared to be about equally potent in the two concentrations. Brand D caused no change in the yield. It should be pointed out, however, that this is a mean result; in two determinations the addition of 10 per cent of brand D caused increases but in two other cases it caused numerically equal decreases, which gave the result indicated in the table. The addition of 10 per cent of brand J caused an insignificant increase. The remaining brands again caused significant and approximately equal decreases.

When the effects of the two concentrations are compared, it will be seen that there is a general relationship. With the fresh juices and with brands A, C and J the two concentrations of juice caused approximately the same increases in yield, with brands E, F, H and I they caused decreases in each case, and only with brands B and D did the effects of the two concentrations show marked differences, in which

cases the increases in yield were significantly less with 10 per cent than with 5 per cent juice added.

It is of considerable interest to note that the color of the tomatoes did not appear to influence the effect of the juice on butter cultures, as the increase caused by the juice from the yellow tomatoes was approximately equal to that caused by the juice from the red tomatoes. This was true for both the concentrations used.

The data presented above make it evident that fresh juices bring about the greatest increases in yield, irrespective of the color of the fruit, but brands of canned tomatoes and of tomato juices vary greatly in their action on butter cultures. It seems logical, therefore, to reason, by analogy, that they may also vary in their suitability for use in tomato juice agar (20) or bouillon (17), where they are used in relatively high concentrations (40 per cent).

In this connection it is of interest to note that brands A and B are those used for the preparation of tomato juice agar and bouillon in the Dairy Bacteriology laboratories at the Iowa State College. These media are very satisfactory for the culture of Streptococcus lactis and the citric acid fermenting streptococci, Streptococcus citrovorus and Streptococcus paracitrovorus.

## Part II

### Relation of Effects of Juices from Various Brands of Tomatoes on Yield of Acetylmethylcarbinol plus Diacetyl by a Butter Culture to Certain Characteristics of the Juices

As the juices used in Part I showed such marked differences in their action on a butter culture, it was decided to study certain of their characteristics to determine whether any relation could be observed between these and their effects on the yield of acetylmethylcarbinol plus diacetyl by a butter culture. Representative samples of the juices used in Part I were examined.

The specific gravity was determined by using a Quevenne lactometer at room temperature (about 21° C.) and correcting the readings to 15.5° C., the temperature for which the instrument was calibrated. The per cent of salt present was determined by titrating 1 ml. of tomato juice, diluted by the addition of 10 ml. of distilled water, with a solution containing 29.06g. of silver nitrate per liter. Potassium chromate was used as the indicator. A blank determination, using 11 ml. of distilled water showed that 0.4 ml. of the silver nitrate solution was required to obtain the same color as that used as the end point, which necessarily was dark because of the color of the tomato juice; this amount was subtracted from all the values obtained. The pH was determined electrometrically. The color was determined by ranging uniform bottles, all containing the same amount of liquid, against a

white background and moving them until they were arranged in order from the lightest, which was designated 1, to the darkest, which was 11. The results of these determinations and some of the data shown in Table I, (the average net increases and the average per cent increases in acetylmethylcarbinol plus diacetyl when 5 per cent of each brand of juice was used) are reported in Table II. For convenience in comparing the effects of the different brands of juice, they are arranged in the table according to their effects on a culture when 5 per cent of each juice was added. The data indicate no relation between the specific gravities of the juices and their effectiveness in increasing the yields of acetylmethylcarbinol plus diacetyl produced by a butter culture.

There was no general relation between the per cent of salt present in a juice and its effect on the yield, but it is of considerable interest to note that three samples, brands E, F and I, which were obtained from canned tomato juice, have salt contents much higher than those obtained from canned, whole tomatoes; these brands caused marked inhibition of production when they were added to a culture. On comparing the salt contents of the juices from fresh tomatoes with those of the canned tomatoes, it is evident that some salt is added during processing. Brand C, which contained the highest salt concentration of the canned whole tomatoes, was described as "flavored with salt", but this concentration of salt did not

TABLE II

RELATION OF EFFECTS OF JUICES FROM VARIOUS BRANDS OF TOMATOES ON YIELD OF ACETYLMETHYLCARBINOL PLUS DIACETYL BY A BUTTER CULTURE TO CERTAIN CHARACTERISTICS OF THE JUICES

Data on effects of juices are from Table I

| Type of juice | Effect of adding juice | | Characteristics of juice | | | |
|---|---|---|---|---|---|---|
| | Net increase in mg. Ni-salt* | Per cent increase in Ni-salt* | Specific gravity | Per cent salt | pH value | Relative color |
| Juice from red tomatoes | 25 | 39.7 | 1.0178 | 0.025 | 4.28 | 3 |
| Juice from yellow tomatoes | 24 | 39.0 | 1.0192 | 0.010 | 4.25 | 8 |
| Brand A | 23 | 37.7 | 1.0228 | 0.450 | 4.26 | 6 |
| Brand B | 23 | 39.0 | 1.0249 | 0.435 | 4.15 | 11 |
| Brand C | 19 | 31.2 | 1.0248 | 0.590 | 4.19 | 5 |
| Brand D | 8 | 13.3 | 1.0225 | 0.315 | 4.23 | 4 |
| Brand J | 5 | 10.0 | 1.0264 | 0.270 | 4.23 | 2 |
| Brand H | - 5 | - 7.7 | 1.0193 | 0.195 | 4.28 | 1 |
| Brand F | - 6 | -12.0 | 1.0218 | 0.900 | 4.06 | 10 |
| Brand E | - 7 | -10.8 | 1.0243 | 0.810 | 4.23 | 9 |
| Brand I | - 7 | -10.8 | 1.0258 | 0.810 | 4.15 | 7 |

*Mg. Ni-salt equivalent to acetylmethylcarbinol plus diacetyl per 200g. culture.

appear to affect,significantly, the effectiveness of the juice in increasing the yield of acetylmethylcarbinol plus diacetyl by a butter culture. There appeared to be no relation between the pH of the juice and its effect on the yield.

The relative color of the samples of juice did not appear to be related to the effect on the yield, but a possible reason for this is that colors were compared after sterilization.

It appears that variations in sterilizing exposures of only a few minutes affect the color markedly. Observations showed that unless the autoclave was opened promptly when the internal pressure became equal to that of the atmosphere a marked darkening occurred even at temperatures below the boiling point. It is possible that the darkening was due, at least in part, to a caramelization and partial hydrolysis of some of the carbohydrate material present.

The characteristics of brand H are of considerable interest. It had a low specific gravity, a low salt content and a high pH value. The addition of water during manufacturing is a possible explanation of these results. Dilution would result in a decrease in specific gravity, a reduction in acidity, in salt content (if none were added to make up for this) and perhaps indirectly in color, since lowered acidity might reduce hydrolysis and formation of colored materials. From the foregoing discussion it appears that the only rela-

tion between the characteristics of the juices from various brands of tomatoes and their effects on the yield of acetylmethylcarbinol plus diacetyl in a butter culture, was that when the salt content was relatively high (0.8-0.9 per cent) the yield was lowered very significantly below that of the cultures to which no juice was added.

While the concentration of salt was a possible explanation of the low yields obtained with three brands, there were other brands which also increased the yield either slightly or not at all and the characteristics studied did not give any explanation for this effect.

The suggestion is made that when juice from commercially canned tomatoes is used for the preparation of tomato juice agar or bouillon it be tested for salt, because high concentrations of salt might inhibit the growth of organisms as well as their activity. If the media are intended for very critical work, it might be advisable to assay the juice biologically, using the juice from fresh tomatoes as a standard.

## Part III

### Effects of Tomato Juice on the Course of the Fermentation of Citric Acid by Butter Cultures

During the earlier work (1) on the effect of tomato juice on butter cultures, it was noted that usually the production of acetylmethylcarbinol plus diacetyl was increased by the added juice, but occasionally a whole series of determinations showed practically no increase, or sometimes an actual decrease, when tomato juice was used. A consideration of these series showed that they had been allowed to incubate some hours longer than the usual period of 14 to 18 hours, and it was also noted that when the incubation period was shorter than normal the yields of the cultures containing tomato juice were very much higher than the controls. When the cultures were incubated for 24 hours and then held at a low temperature for another 24 hours, the presence of tomato juice either produced only very slight increases or actually decreased the yield, although the controls contained more acetylmethylcarbinol plus diacetyl than those incubated for 24 hours.

The results suggested that the production of acetylmethylcarbinol plus diacetyl was accelerated by the addition of tomato juice to cultures, but it seemed that finally the cultures which did not contain tomato juice produced substantially the same amounts as those which did contain tomato juice.

To obtain more detailed data regarding the action of tomato juice on the yields of acetylmethylcarbinol plus diacetyl by butter cultures, examinations of the cultures were made at frequent intervals in order to follow changes in the amounts of these compounds present.

In carrying out each trial, a comparatively large volume of milk, the exact amount depending on the number of analyses to be made, was prepared and inoculated as usual. Portions of this milk, each consisting of 250 ml., were poured into sterile cotton stoppered bottles. Enough bottles were provided so that there would be one for each period at which analyses were to be made. To the remainder of the milk, sufficient tomato juice was added to equal 5 per cent of the total volume. This inoculated milk, in 250 ml. portions, was also poured into a series of bottles. All the portions were incubated at 21° C. The first determinations were made 10 or 16 hours after inoculation and thereafter determinations were commonly made every 2 hours.

Each determination was made on a separate bottle of milk. Conditions in the various bottles may have varied somewhat, even though attempts were made to keep them as uniform as possible. It was felt, however, that this method introduced less variation than would occur if a large volume of culture were prepared and samples taken from this at intervals. The

agitation necessary to mix the culture thoroughly so that a representative sample could be obtained would introduce considerable quantities of air. The presence of more than the normal amount of air might result in the oxidation of acetylmethylcarbinol to diacetyl and retard its reduction to 2,3-butylene glycol, thus yielding results which would be too high.

The results obtained in several experiments using different cultures are given in Table III and the data on two cultures which were examined at intervals over a period of 18 hours are presented in Figure I.

A study of the data shows that the cultures analyzed 10 hours after inoculation contained only relatively small amounts of acetylmethylcarbinol plus diacetyl. During the next 10 to 14 hours the amount present in all cultures increased rapidly and reached a maximum 18 to 26 hours after inoculation. After the maximum yield was reached a gradual decrease in the amount of acetylmethylcarbinol plus diacetyl occurred. It will be seen that those cultures with added tomato juice contained very definitely more acetylmethylcarbinol plus diacetyl at the time of the first determination and that the amount present increased more rapidly than it did in the controls. The former attained the maximum content in 18 to 24 hours after inoculation while the controls did not reach their

TABLE III

EFFECT OF TOMATO JUICE ON YIELD OF ACETYLMETHYLCARBINOL PLUS DIACETYL BY BUTTER CULTURES

| Hours after inoc. | Mg. Ni-salt* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Culture F | | Culture F | | Culture F | | Culture 15-3 | | Culture OD | |
| | Control | 5% Juice | Control | 5% Juice | Control | 5% Juice | Control | 5% Juice | Control | 5% Juice |
| 10 | 4.4 | 7.6 | | | | | 6.4 | 7.6 | 6.6 | 11.6 |
| 12 | 8.1 | 14.7 | | | | | 13.7 | 19.5 | 17.2 | 30.6 |
| 14 | 18.5 | 32.3 | | | | | 37.1 | 46.3 | 35.7 | 63.2 |
| 16 | 32.6 | 54.6 | 47.0 | 55.6 | 49.7 | 69.0 | 62.5 | 74.7 | 50.2 | 89.5 |
| 17 | | | 62.1 | 72.5 | 66.9 | 87.2 | | | | |
| 18 | 48.8 | 80.0 | | | | | 91.2 | 111.0 | 72.6 | 105.3 |
| 19 | | | 73.2 | 84.1 | 78.1 | 87.7 | | | | |
| 20 | 52.9 | 91.2 | | | | | 119.7 | 119.7 | 94.4 | 100.5 |
| 21 | | | 80.8 | 78.8 | - | - | | | | |
| 22 | 67.5 | 85.6 | | | | | 122.5 | 116.2 | 103.8 | 102.7 |
| 23 | | | 76.4 | 75.7 | 86.7 | 82.0 | | | | |
| 24 | 83.4 | 83.4 | | | | | 117.2 | 120.7 | 99.5 | 100.9 |
| 25 | | | 77.2 | 75.0 | 79.4 | 80.0 | | | | |
| 26 | | | | | | | 115.0 | 106.7 | 107.8 | 94.7 |
| 27 | | | 73.3 | 74.0 | 82.5 | 79.0 | | | | |
| 28 | | | | | | | 110.7 | 95.3 | 98.5 | 99.6 |
| 29 | | | 70.0 | 72.4 | 80.2 | 73.3 | | | | |

*Mg. Ni-salt equivalent to acetylmethylcarbinol plus diacetyl per 200 g. culture.

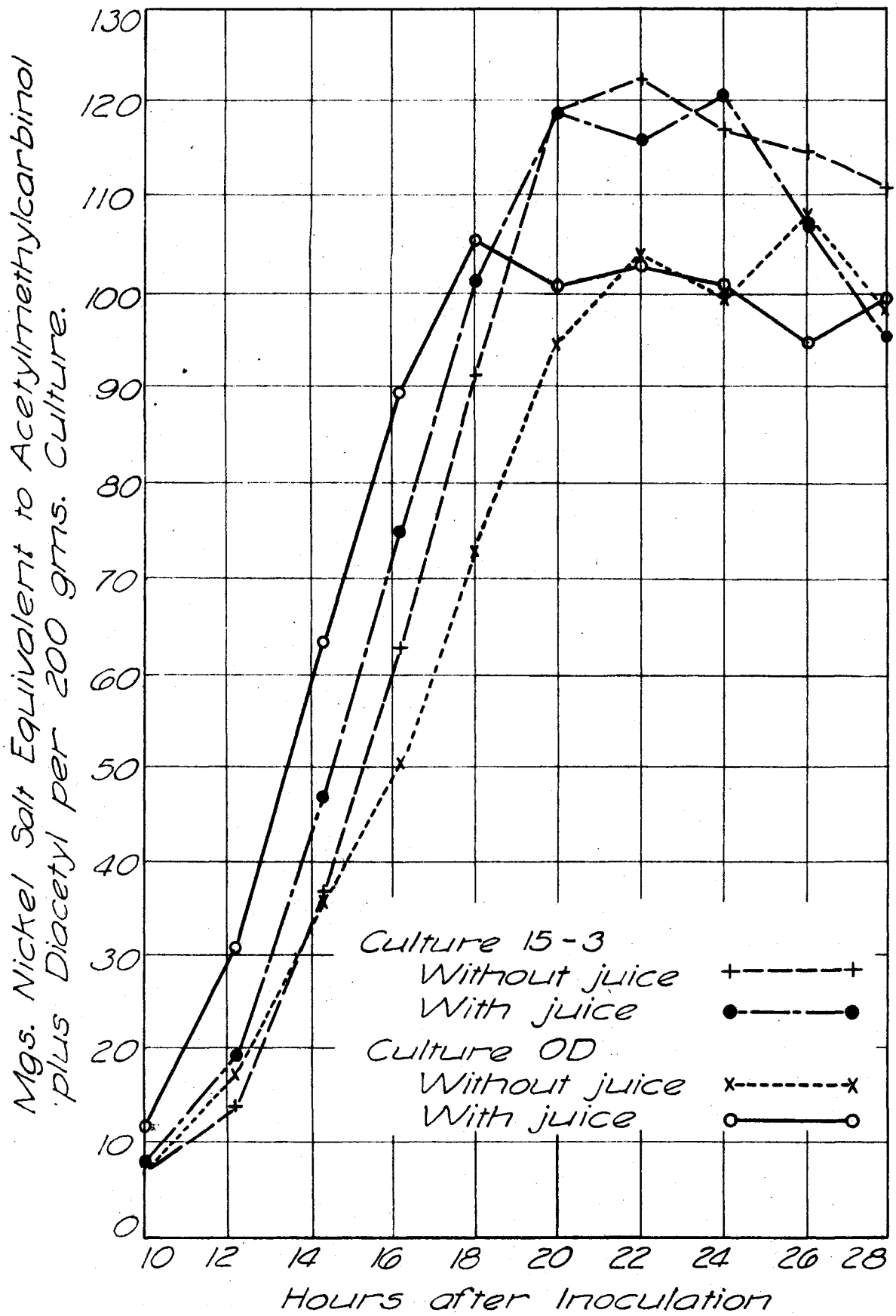


Fig. I. Effects of tomato juice on the yield of acetylmethylcarbinol plus diacetyl by butter cultures.

maximum until 21 to 26 hours had elapsed.

Most striking fact of all is that, while production was much more rapid in those cultures containing tomato juice than in the controls, the maximum values are about the same with and without tomato juice. In the trials shown in Table III the maximum values of the cultures containing tomato juice exceeded those of the corresponding controls by an average value of only 1.7 mg. which is not significant.

From the foregoing data it is evident that tomato juice had a marked acceleratory action on the production of acetylmethylcarbinol plus diacetyl in butter cultures, since it increased the rate of production of these materials but did not significantly increase the total amounts formed.

As the maximum yield of acetylmethylcarbinol plus diacetyl was not increased by the addition of tomato juice, although the rate of production was greatly increased, it appears that the tomato juice did not furnish additional amounts of substrate from which these substances could be formed, but instead increased the action of the culture. The increased activity may have resulted from a more rapid increase in the number of organisms present, or from a more rapid fermentation of citric acid by essentially the same numbers of organisms as were present in the cultures containing tomato juice.

## Part IV

### Comparison of Effects of Tomato Juice on Yield of Acetylmethylcarbinol plus Diacetyl by Different Butter Cultures

During the earlier stages of this work (1) it was noted that butter cultures varied markedly in their response to the addition of tomato juice. Cultures which normally produced low yields of acetylmethylcarbinol plus diacetyl showed considerable increases when tomato juice was added, while cultures which produced comparatively large amounts of these materials either showed only very slight increases or else the presence of tomato juice actually decreased the yield.

At the time, this was ascribed to the fact that the yield was limited by the amount of citric acid present and that the maximum yield that could be obtained, when 0.15 per cent citric acid was added, was about 100 mg. of Ni-salt. High yielding cultures were believed to be those in which a comparatively large proportion of the citric acid present was fermented to acetylmethylcarbinol plus diacetyl, while low yielding cultures were those in which either less citric acid was fermented or the same amount was fermented but a greater proportion of it was changed to carbon dioxide, acetic acid and 2,3-butylene glycol so that less acetylmethylcarbinol plus diacetyl was formed. Thus, in a high yielding culture the tomato juice could not increase the yield significantly because a maximum yield of about 100 mg., of Ni-salt was already

being obtained from the citric acid present. When the yield of a culture was low the addition of tomato juice could increase it up to the maximum of about 100 mg. of Ni-salt, either by increasing the total amount of citric acid fermented or else by increasing the ratio of acetylmethylcarbinol plus diacetyl to carbon dioxide, acetic acid and 2,3-butylene glycol.

In the light of the data reported in Part III, it appears, however, that the addition of tomato juice to a culture does not influence the total amount of acetylmethylcarbinol plus diacetyl formed, but does have a pronounced effect on the rate of production of these substances. A more likely explanation of the fact that the yields of some cultures were increased by the addition of tomato juice, while the yields of others were not significantly changed, or else decreased, is that the cultures in which the presence of tomato juice increased the yield were analyzed after a relatively short incubation period. Those cultures in which the presence of juice did not increase the yield probably were analyzed during the period when the cultures with and without juice contained approximately the same amounts of acetylmethylcarbinol plus diacetyl.

In the earlier work (1) cultures were usually inoculated about 5 p.m. and analyzed between 9 a.m. and 12 noon the next day, which gave an incubation period of 16 to 19 hours. As

the importance of the incubation period was not realized at the time the experiments were conducted, inoculations were occasionally made as early as 2 p.m. and analyses not started until 10 a.m., thus making the incubation period about 20 hours.

Reference to Table III and Figure I shows that at the end of the usual incubation period, 15 to 18 hours, the cultures containing tomato juice showed distinct increases in yield over those to which juice was not added, but after 20 to 24 hours the differences were much less marked.

When it was understood that the addition of tomato juice did not increase the yield of acetylmethylcarbinol plus diacetyl but rather increased the rate of production, it was decided to determine whether cultures differed in their response to tomato juice. Eleven cultures were analyzed at various intervals after inoculation to compare their response during the period when the amount of acetylmethylcarbinol plus diacetyl was being produced rapidly with their response during the period when the yields of the cultures were approximately at their peaks.

Because of the work involved, only six cultures could be compared at one time, thus each trial required two separate runs to furnish the information. To carry out the test, 6.0 liters of milk were pasteurized and cooled, 0.15 per cent of citric acid was added and the milk was mixed thoroughly.

Twenty ml. of each culture were added to 1000 ml. portions of the milk and thoroughly distributed. Each of these portions was divided into two equal parts. To one was added 5 per cent of tomato juice while the other was used as a control. The cultures containing tomato juice and the controls were then again divided into two equal portions. One portion was examined 14 hours and the other 18 hours after inoculation. The results obtained are reported as trials 1 and 4 in Table IV. In an attempt to gain an idea of the approximate time at which peak production occurred, another trial was run in which each culture was analyzed 17 hours after inoculation only, (not at 14 hours as well). The data are shown as trial 2 in table IV. To compare the response of the same culture at different propagations a fourth trial was made in which the cultures were incubated for 18 hours; this is reported as trial 3 in Table IV.

The table shows that after incubating 14 hours all those cultures to which tomato juice had been added contained more acetylmethylcarbinol plus diacetyl than did the controls. The increases ranged from 11.2 to 34.0 mg.; two* were relatively small (11.2 and 13.8 mg.), one was very considerable (34.0) while the other eight varied between 21.6 and 29.0 mg.

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*Of the two low cultures, 122-F is regarded as being unsatisfactory for use as a butter culture, and H-6 is used only as a cheese culture.

TABLE IV

DIFFERENCES IN RESPONSE OF BUTTER CULTURES TO ADDITION OF TOMATO JUICE

Cultures are listed according to the increase in acetylmethylcarbinol plus diacetyl 14 hours after inoculation

| Culture number | Mg. Ni-salt* | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Trial 1, 14 hours after inoculation | | | Trial 2, 17 hours after inoculation | | | Trial 3, 18 hours after inoculation | | | Trial 4, 18 hours after inoculation | | |
| | Control | 5 % Juice | Diff. in yield* | Control | 5 % Juice | Diff. in yield* | Control | 5 % Juice | Diff. in yield* | Control | 5 % Juice | Diff. in yield* |
| 122-F | 57.8 | 68.0 | 11.2 | - | - | - | 72.3 | 93.2 | +10.9 | 70.3 | 92.2 | +21.9 |
| H6 | 48.8 | 62.6 | 13.8 | - | - | - | 77.8 | 71.8 | - 6.0 | 71.0 | 90.1 | +19.1 |
| 15-3 | 40.7 | 62.3 | 21.6 | 84.0 | 113.2 | +29.2 | 75.2 | 75.7 | + 0.5 | 117.6 | 120.2 | + 2.6 |
| 103 | 63.0 | 84.7 | 21.7 | 65.4 | 87.9 | +22.5 | 88.7 | 75.6 | -13.1 | 76.0 | 73.2 | - 2.8 |
| 15-1 | 55.9 | 80.2 | 24.3 | 77.7 | 77.1 | - 0.6 | 103.6 | 106.2 | + 2.6 | 88.9 | 85.5 | - 3.4 |
| OD | 36.0 | 61.4 | 25.4 | 61.3 | 96.3 | +35.0 | 68.0 | 84.2 | +16.2 | 111.5 | 111.8 | + 0.3 |
| F | 59.6 | 86.1 | 26.5 | 81.5 | 88.5 | + 7.0 | 89.1 | 78.9 | -10.2 | 76.6 | 89.8 | +13.2 |
| A | 44.9 | 73.0 | 28.1 | 69.9 | 81.9 | +12.0 | 88.8 | 86.3 | - 2.5 | 83.3 | 79.2 | - 4.1 |
| 232 | 42.1 | 70.5 | 28.4 | 68.2 | 77.1 | + 8.9 | 82.7 | 81.8 | - 0.9 | 82.7 | 80.8 | - 1.9 |
| 233 | 33.8 | 62.8 | 29.0 | 70.4 | 81.2 | +10.8 | 80.9 | 78.8 | - 2.1 | 62.8 | 85.3 | +22.5 |
| 146 | 56.2 | 90.2 | 34.0 | 86.3 | 79.7 | - 6.6 | 87.9 | 84.0 | - 3.9 | 95.7 | 99.6 | + 3.9 |

*Mg. Ni-salt equivalent to acetylmethylcarbinol plus diacetyl per 200g. culture

When nine of the same cultures were incubated 17 hours and then analyzed, it was found that seven still showed increases, one showed practically no difference (a decrease of 0.6 mg.) and one showed a decrease of 6.6 mg. Thus after 17 hours, in the majority of cultures, the presence of tomato juice still accelerated the production, although in one culture the control had reached the same level and in another the destruction of acetylmethylcarbinol plus diacetyl had apparently begun.

Two trials were made when cultures were incubated for 18 hours and then analyzed, and the results are very variable. Culture 122-F, the yield of which was increased the least by tomato juice during a 14 hour incubation period, showed definite increases in each trial, culture OD showed a significant increase in only one case, 15-3 showed negligible increases, and the other cultures either showed decreases in both cases or an increase in one and a decrease in the other.

While the above experiment gives considerable information as to the variability of the response of different cultures to tomato juice, it was decided to study this in greater detail by comparing the course of the fermentation of citric acid, with and without added juice. Table III and Figure I include data for two cultures, 15-3 and OD, which permit such a consideration.

On referring to the table and figure it will be seen that the two controls start at approximately the same value, but

culture 15-3 produced acetylmethylcarbinol plus diacetyl more rapidly and gave higher yields than did culture OD. The former reached a peak some hours before the latter, but on studying Figure I it will be seen that the rapid increases in the control cultures ceased at about the same time. The minor deviations after the rapid increases ceased are probably the result of experimental errors.

On comparing the two cultures to which tomato juice had been added it is evident that they differ greatly in their reaction to the juice. The rate of production of acetylmethylcarbinol plus diacetyl by culture 15-3 was only slightly increased and after 20 hours both the control and the sample with added juice contained exactly the same amount of these materials. During the next 2-hour period the control increased slightly and after this, both showed a gradual decrease. Culture OD showed a much greater response to the presence of tomato juice and the sample containing juice reached a peak value some 4 hours before the control.

The above experiment shows that under controlled conditions in which equal amounts of culture were inoculated into the same milk containing the same amounts of citric acid, two cultures will differ markedly in their production of acetylmethylcarbinol plus diacetyl and in their response to the presence of tomato juice.

It appears, however, that under normal experimental con-

ditions, differences in yield may occur between different propagations of the same culture which are quite as great as those which occur between simultaneous inoculations of two different cultures into the same milk.

Tables III and IV include data on the responses of several cultures to the addition of 5 per cent tomato juice. The increases in the yields of acetylmethylcarbinol plus diacetyl in two propagations of each of three cultures, at various intervals after inoculation, are summarized below. The values are the increases in the yields of the cultures containing tomato juice, over the controls.

Increase in Yields of Acetylmethylcarbinol plus Diacetyl by Butter Cultures on Addition of 5 Per Cent Tomato Juice

| Hours after inoculation | Mg. Ni-salt* | | | | | |
|---|---|---|---|---|---|---|
| | Culture F | | Culture 15-3 | | Culture OD | |
| | Trial I | Trial II | Trial I | Trial II | Trial I | Trial II |
| 14 | 13.8 | 26.5 | 21.6 | 9.2 | 27.4 | 25.4 |
| 17 | 27.0 | 7.0 | 29.2 | 10.5 | 35.5 | 35.0 |
| 18 | 31.2 | 13.2 | 2.6 | 19.8 | 32.8 | 16.2 |

*Mg. Ni-salt equivalent to acetylmethylcarbinol plus diacetyl per 200g. culture.

With culture OD the increases with two of the three incubation periods agreed very well in the two trials, but at the third period the increases differed considerably and this lack of agreement was evident at all three incubation periods with the other two cultures. The results indicate that although attempts were made to control experimental conditions

and treat the various propagations of a culture in the same way, there was considerable variation in the response of a culture to tomato juice.

Possible causes of these differences in response at various propagations of a culture include variations in the amount of citric acid naturally present in the milk and variations in the distribution of the added citric acid, for although it was added carefully, slight curdling occurred and the lumps of curd undoubtedly occluded some of the citric acid. Variations in the amount and activity of the inoculum used and in the temperature of incubation may also have been involved in the discrepancies noted.

The data presented above indicate that butter cultures show marked differences in their responses to the addition of tomato juice and also that differences which occur between two propagations of the same culture may be as great as those observed between two cultures inoculated simultaneously into milk from the same lot.

## Part V

## Comparison of Effects of Neutralized and Untreated Tomato Juices on Yield of Acetylmethylcarbinol plus Diacetyl by Butter Cultures

### A. Neutralization with Calcium Carbonate

In the preliminary studies (1) it was observed that the addition of certain concentrations (1 per cent to 20 per cent) of tomato juice increased the yields of acetylmethylcarbinol plus diacetyl by butter cultures, but these increases were not proportional to the amounts of juice added; higher concentrations (30 per cent or 40 per cent) either caused smaller increases than the lower concentrations or else lowered the yields below those of the cultures without juice.

The increases in yield caused by the lower concentrations of juice may have resulted from the increase in initial acidity caused by the addition of an acidic material. The higher initial acidity may have meant that a pH satisfactory for the rapid production of acetylmethylcarbinol plus diacetyl by butter cultures was reached sooner than in the control cultures. In cultures without added tomato juice, the optimum pH for the production of acetylmethylcarbinol plus diacetyl would have to be developed through the lactic acid elaborated by organisms while in cultures containing added juice, the juice would contribute a small amount of acid. The reduction in yield observed when a relatively large amount of juice was added may have been due to the fact that the tomato

juice, containing about 0.15 per cent citric acid (34), replaced an equivalent volume of milk which contained about 0.33 per cent citric acid (0.18 per cent naturally present (31) and 0.15 per cent added). Another possibility is that the addition of a large quantity of the juice to a butter culture seriously upset the buffer capacity of the milk.

As the pH of the medium has such a marked influence on the fermentation of citric acid by the citric acid fermenting streptococci (26, 28,35), the effect of the acidity of the juice was studied by comparing the effects of neutralized and untreated tomato juices on the production of acetylmethylcarbinol plus diacetyl by butter cultures.

Tomato juice was neutralized by shaking for several hours with an excess of powdered calcium carbonate and then centrifuging the mixture to remove the excess carbonate and the other solid material. A clearer solution could be obtained in this way than by filtering. The pH values of three samples of juice before neutralizing were 4.15, 4.16 and 4.32; after treatment the values were 6.62, 6.23 and 6.42, respectively, indicating that a considerable reduction in acidity had been effected.

Neutralized juice was added to cultures in various concentrations and the increases in yield of acetylmethylcarbinol plus diacetyl were compared with those produced in cultures to which untreated juice was added. In order to obtain cul-

tures in which the initial acidity would be as low as possible, sodium citrate was added to the milk in one series of experiments, while citric acid was used in the control series.

In the control series, neutralized juice was added in amounts ranging from 1 per cent to 40 per cent to milk prepared in the usual way with culture 15-1. Juice from the same lot which had not been treated with calcium carbonate was added to cultures prepared from the same inoculated milk. The cultures were held overnight and then the amounts of acetylmethylcarbinol plus diacetyl present were determined. The data on the three trials are presented in Table V and the average values are shown in Figure II.

The general results indicate that both types of juice produced increases in all concentrations, but those obtained when 40 per cent of the juices were added were not as great as those obtained when certain lower concentrations were used. This is in agreement with the results obtained in the preliminary studies. On the basis of average results, concentrations of neutralized tomato juice up to 10 per cent produced greater increases in yield than the untreated juice. In concentrations of 20 per cent and 40 per cent, however, the untreated juice had a greater effect on the yield, although both types of juice produced much smaller increases in the latter concentration than in the former. All of the trials except one, showed a maximum production of acetylmethylcar-

TABLE V

EFFECTS OF VARIOUS CONCENTRATIONS OF NEUTRALIZED AND UNTREATED TOMATO JUICES ON YIELD OF ACETYLMETHYLCARBINOL PLUS DIACETYL BY BUTTER CULTURES WHEN CITRIC ACID WAS ADDED

Cultures incubated 16-20 hours.

| Trial no. | Juice | Mg. Ni-salt* | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | No juice | 1 % juice | 3 % juice | 5 % juice | 10 % juice | 20 % juice | 40 % juice |
| I | Untreated | 47.2 | 45.2 | 55.5 | 62.1 | 73.8 | 90.8 | 60.3 |
| | Neutralized | 47.2 | 46.8 | 72.8 | 78.1 | 82.2 | 83.3 | 56.5 |
| II | Untreated | 36.5 | 43.6 | 63.3 | 72.5 | 85.9 | 88.0 | 60.6 |
| | Neutralized | 36.5 | 54.3 | 67.2 | 83.2 | 86.2 | 70.4 | 47.3 |
| III | Untreated | 35.1 | 42.8 | 59.8 | 71.4 | 80.1 | 84.7 | - |
| | Neutralized | 35.1 | 49.5 | 71.9 | 81.0 | 82.0 | 88.8 | 60.2 |
| Average results | Untreated | 39.6 | 43.8 | 59.5 | 68.7 | 79.9 | 87.8 | 60.4 |
| | Neutralized | 39.6 | 50.2 | 70.6 | 80.7 | 83.4 | 80.8 | 54.6 |

| | Average increase for each 1 per cent juice added | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 % | 2 % | 3 % | 5 % | 10 % | 20 % | 40 % |
| Untreated | 6.7 | 5.7 | 7.4 | 6.3 | 4.3 | 2.53 | 0.58 |
| Neutralized | 10.7 | 10.4 | 10.3 | 8.2 | 4.3 | 2.06 | 0.43 |

*Mg. Ni-salt equivalent to acetylmethylcarbinol plus diacetyl per 200g. culture.

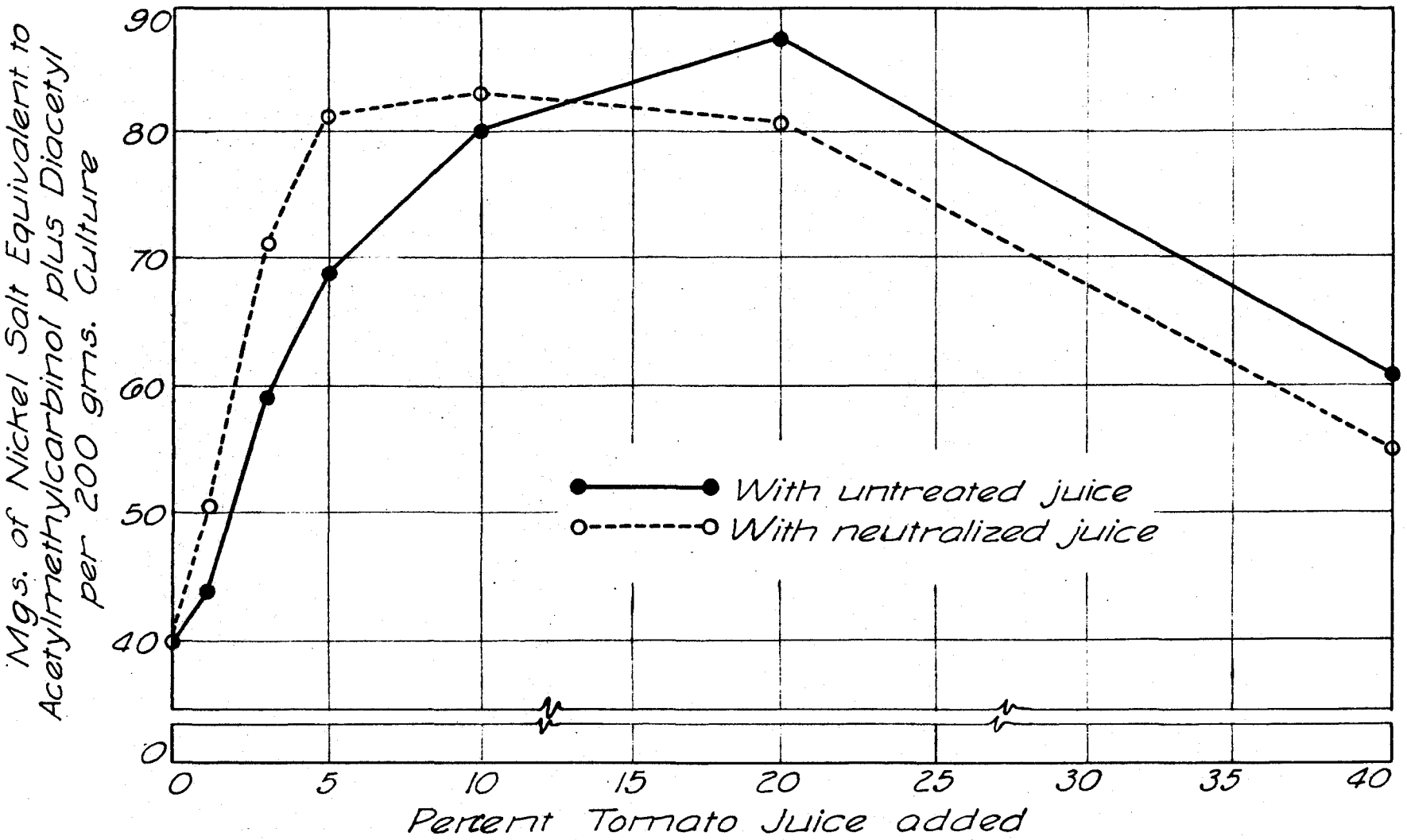


Fig. II. Effects of various concentrations of neutralized and untreated tomato juice on yield of acetylmethylcarbinol plus diacetyl by butter cultures when citric acid was added.

binol plus diacetyl when 20 per cent of juice was present. Table V includes data which show the results obtained when the average increases in yield, produced by the various concentrations of tomato juice, are divided by the corresponding per cent of tomato juice added, to obtain the increase per 1 per cent of juice at the various levels. The values given for 2 per cent were determined from the figure. While they are theoretical values, they are added for purposes of comparison. The results show that up to 5 per cent the neutralized juice was more efficient in increasing the yield of acetylmethylcarbinol plus diacetyl than the untreated juice, that is, it produced greater increases for each per cent added. It will also be noted that the increases for each per cent of both types of juice are rather uniform, in concentrations up to 5 per cent. At concentrations of 10 per cent, the two types of juice were equally efficient but less so than in the lower concentrations, while at concentrations of 20 per cent and 40 per cent the efficiencies of both types of juice were relatively low.

In order to reduce the initial acidity of cultures to as low a level as possible so that the effect of the acidity of the tomato juice could be studied without the complicating effects due to the acidity of added citric acid, sodium citrate was used. The activity of neutralized and of untreated juices was compared in cultures containing this ma-

terial.

To prepare the sodium citrate solution, 50g. of the hydrated salt, 2 $Na_3C_6H_5O_7.11H_2O$ were dissolved and made up to 100 ml. with distilled water. In order to add an amount of citrate radicle equivalent to that obtained by adding 0.15 per cent of citric acid, it was necessary to add 0.26 per cent of the sodium salt. Except for the fact that sodium citrate solution was used in place of citric acid solution, this experiment was carried out in exactly the same way as the previous one and the same culture, 15-1, was used. The results are shown in Table VII and the means in Figure III.

On comparing Tables VI and VII it will be seen that, when sodium citrate was used as a source of citrate radicle, the yields of acetylmethylcarbinol plus diacetyl showed rather wider variations.

On comparing Figures II and III it will be seen that when citric acid was used (Figure II) 40 per cent of both types of juice caused relatively low increases in yield. When sodium citrate was used (Figure III), the culture containing 40 per cent of untreated juice contained the maximum amount of acetylmethylcarbinol plus diacetyl, and although the yield of the culture containing 40 per cent of neutralized juice was less than that of the culture containing 20 per cent, yet the reduction was not as marked as in the series in which citric acid was used.

TABLE VI

EFFECTS OF VARIOUS CONCENTRATIONS OF NEUTRALIZED AND UNTREATED TOMATO JUICES ON YIELD OF ACETYLMETHYLCARBINOL PLUS DIACETYL BY BUTTER CULTURES WHEN SODIUM CITRATE WAS ADDED

Cultures incubated 16-20 hours.

| Trial no. | Juice | Mg. Ni-salt* | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | No juice | 1 % juice | 3 % juice | 5 % juice | 10 % juice | 20 % juice | 40 % juice |
| I | Untreated | 36.6 | 51.1 | 59.7 | 61.6 | 67.6 | 67.6 | 68.9 |
| | Neutralized | 36.6 | 60.8 | 60.1 | 50.1 | 53.6 | 46.3 | 30.7 |
| II | Untreated | 22.5 | 40.7 | 50.4 | 60.1 | 59.9 | 70.9 | 74.8 |
| | Neutralized | 22.5 | 52.3 | 59.2 | 67.0 | 68.9 | 60.8 | 58.6 |
| Average results | Untreated | 29.5 | 45.9 | 55.0 | 60.8 | 63.7 | 69.2 | 71.8 |
| | Neutralized | 29.5 | 56.5 | 59.6 | 58.6 | 61.2 | 53.0 | 44.6 |

| | Average increase for each 1 per cent juice added | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 % | 2 % | 3 % | 5 % | 10 % | 20 % | 40 % |
| Untreated | 14.4 | 10.2 | 7.8 | 6.2 | 3.4 | 1.98 | 1.05 |
| Neutralized | 27.0 | 14.0 | 10.0 | 5.8 | 4.2 | 1.17 | 0.38 |

*Mg. Ni-salt equivalent to acetylmethylcarbinol plus diacetyl per 200g. culture.

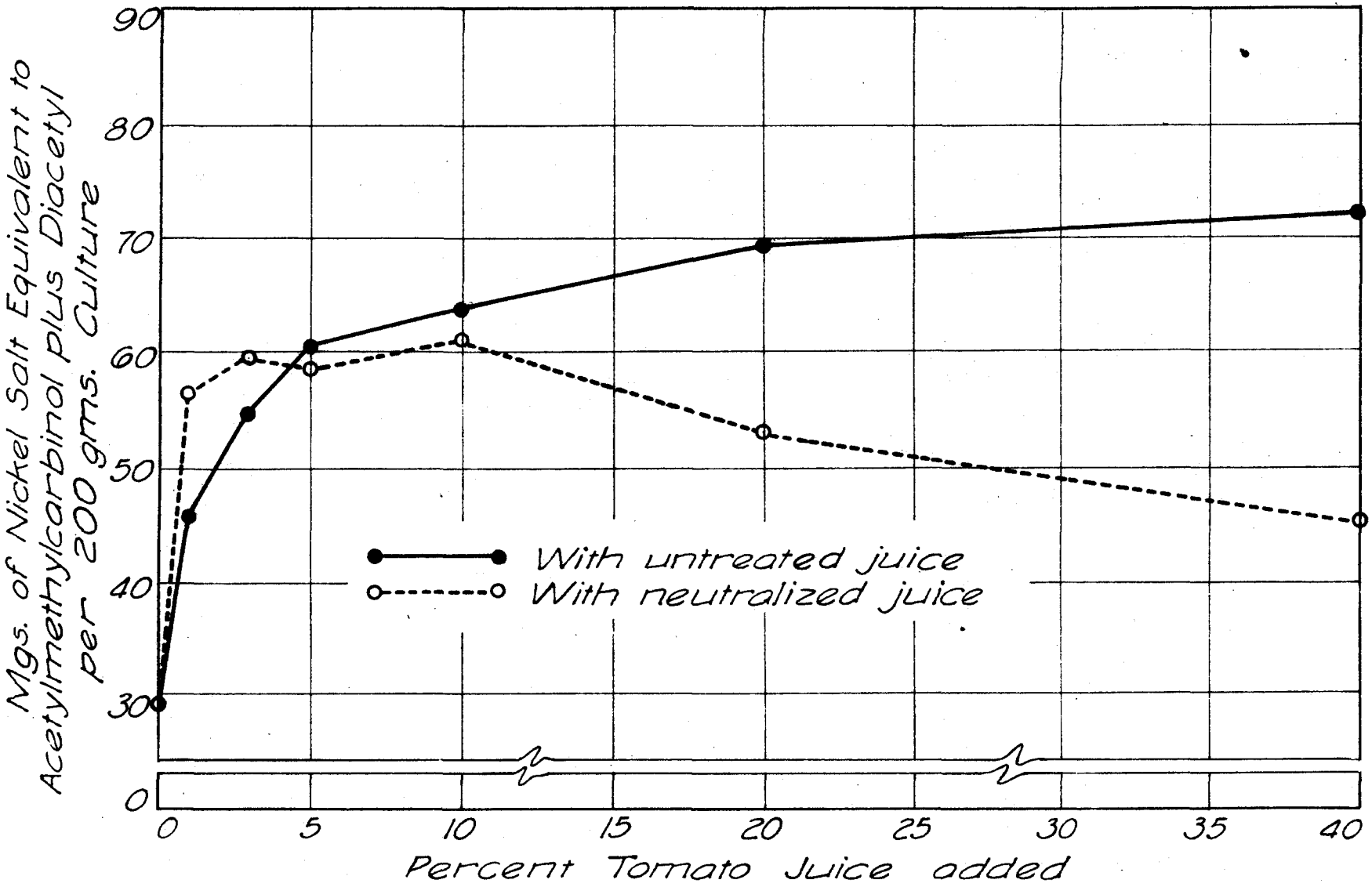


Fig. III. Effects of various concentrations of neutralized and untreated tomato juices on yield of acetylmethylcarbinol plus diacetyl by butter cultures when sodium citrate was added.

When tomato juice was present in concentrations up to 3 per cent in cultures containing sodium citrate, the yields of the cultures containing neutralized juice were greater than those of the cultures containing untreated juice, but above 3 per cent untreated juice produced greater increases than neutralized juice.

The yields obtained when sodium citrate was used were, in general, lower than those obtained with the corresponding concentrations of juice in cultures containing citric acid. A possible explanation of this difference is that, at the time the determinations were made, the acidity of the cultures containing neutralized juice may have been lower than that at which the most rapid production of acetylmethylcarbinol plus diacetyl occurred.

The addition of neutralized juice to a butter culture containing sodium citrate may have diluted the acidity resulting from the formation of lactic acid (practically the only source of acidity in the culture) so that the pH of the medium may not have reached a level at which the most rapid production of acetylmethylcarbinol plus diacetyl occurred. When untreated juice was added, however, the acidity of the juice together with that resulting from the lactic acid formed, probably reduced the pH to a level at which more rapid production of acetylmethylcarbinol plus diacetyl occurred.

In cultures containing citric acid, there was an addition-

al source of acid, the acidity of the added citric acid, so that the situation was different from that when sodium citrate was added. In the more acid medium the presence of high concentrations of juice apparently affected the yield considerably.

From the foregoing discussion it appears that neutralized tomato juice increased the yield of acetylmethylcarbinol plus diacetyl by a butter culture. The lowered increases obtained when high concentrations of juice were added to butter cultures containing added citric acid may depend on the acidity of the culture.

B. Attempts to Remove Citric Acid from Tomato Juice

Attempts were made to remove the citric acid from tomato juice by precipitating the acid as a practically insoluble salt in order to determine whether the citric acid present in the juice had any effect on the yield of acetylmethylcarbinol plus diacetyl by butter cultures.

The following modification of the Denige-Beau method (34) was used to test for the presence of citric acid before and after treatment: 1 ml. of the juice to be examined was placed in a test tube, the same volume of Denige's Reagent (5 per cent mercuric sulphate in 10 per cent sulphuric acid) was added and the mixture boiled. A saturated potassium permanganate solution was added, a drop at a time, until a pre-

cipitate formed, and then a slight excess was added. The mixture was again heated to boiling and the residue of manganese dioxide was removed by adding hydrogen peroxide until the precipitate was colorless. A copious white precipitate indicated the presence of citric acid in the original juice.

Attempts were made to remove the citric acid from samples of juice by treating them with calcium carbonate, barium carbonate and barium hydroxide but in each case, after the mixture was centrifuged to remove the excess reagent and any precipitate formed, the supernatant liquid still gave a positive citric acid reaction. This indicated that citrate still remained in solution. It was therefore not possible to determine, by this method, to what extent the citric acid added in the juice is responsible for the increase observed in the rate of production of acetylmethylcarbinol plus diacetyl when tomato juice was added to butter cultures.

## Part VI

## Effects of Tomato Juice on Activities of the Two Types of Organisms Present in Butter Cultures

The previous work has shown that tomato juice has a pronounced acceleratory effect on the production of acetylmethylcarbinol plus diacetyl by butter cultures. The present study was intended to show the action of tomato juice on the constituent organisms by determining whether the activity of one or both was increased.

The constituent organisms were isolated from butter cultures. The S. lactis cultures were obtained by repeated plating and picking of colonies into litmus milk. Identity of the organisms was established by morphology, growth in litmus milk and a negative creatine test (13). The citric acid fermenting Streptococcus used was isolated by plating a butter culture on tomato juice agar containing α-bromopropionic acid, which largely prevents the development of colonies of S. lactis but not of the citric acid fermenting organisms (29). The identity of the culture selected was established by morphology, growth in litmus milk and fermentation of citric acid with the production of acetylmethylcarbinol plus diacetyl.

### A. Effects on the Production of Acid in Milk by S. lactis

Three strains of S. lactis were used; they were isolated

from butter cultures OD, 15-3 and 232. These organisms were carried, with frequent transfers, for about 2 weeks in litmus milk and were then inoculated into sterilized milk and sterilized milk containing 5 per cent tomato juice. The cultures were incubated for 10 hours at 21° C. and then the total acidity and pH were determined every 2 hours for the next 18 hours.

To determine the amount of acid produced, the flasks were shaken thoroughly to break up the curd. Then 10 ml. portions were removed by means of a pipette and the outside of the pipette was carefully wiped. The contents were permitted to run into a porcelain casserole and the pipette was thoroughly rinsed with from 30 to 40 ml. of distilled water. This water not only removed the milk adhering to the pipette but also served to dilute the milk so that the slight brown color, due to sterilization, did not interfere with the end point (39). This dilution undoubtedly lowered the titration value slightly by disturbing the balance of mono- and dibasic phosphates, but as approximately the same amount of water was used in each determination this should not interfere with the relationships of the various values obtained. A 1 per cent alcoholic solution of phenolphthalein was used as the indicator. Titration with 0.1 N sodium hydroxide was continued until a faint, but definite, pink persisted throughout the sample. The values were converted to per cent lactic acid

and are reported in Table VII. The means of the three trials are shown in Figure IV.

The individual trials, whether with or without tomato juice, varied, but in general they showed the same tendency: a period of rapid increase in the per cent lactic acid present, followed by a period in which the acid content was relatively constant and then a slight reduction in the amount of acid present which was more definite in the case of the controls.

By studying the mean values as shown in Figure IV it is somewhat easier to determine the influence of tomato juice as the effect of experimental errors is minimized. The titrable acidity of the control cultures increased rapidly until 18 hours after inoculation. For the next 4 hours the acidity was practically constant but after this it showed a slight decline. The initial titrable acidity of the cultures containing tomato juice was higher than that of the controls, presumably because of the acidity of the tomato juice. The acid production in these cultures was less rapid than in the controls, and both reached approximately the same peak value about 20 hours after incubation. After this the acidity of the cultures containing tomato juice remained practically constant. The pH values of the control culture showed a rapid decrease until 18 hours after inoculation and then remained fairly constant. The pH values of the cultures containing tomato juice followed the same course as those of the

TABLE VII

EFFECTS OF TOMATO JUICE ON ACID PRODUCTION IN MILK BY S. LACTIS

| Hours after inoc. | S. lactis OD* | | | | S. lactis 15-3* | | | | S. lactis 232* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control | | 5 % juice | | Control | | 5 % juice | | Control | | 5 % juice | |
| | % Lactic acid | pH | % Lactic acid | pH | % Lactic acid | pH | % Lactic acid | pH | % Lactic acid | pH | % Lactic acid | pH |
| 10 | 0.619 | 4.52 | 0.744 | 4.53 | 0.624 | 4.70 | 0.731 | 4.55 | 0.713 | 4.55 | 0.782 | 4.45 |
| 12 | 0.722 | 4.45 | 0.782 | 4.50 | 0.688 | 4.62 | 0.787 | 4.51 | 0.800 | 4.47 | 0.817 | 4.45 |
| 14 | 0.816 | 4.44 | 0.816 | 4.44 | 0.726 | 4.55 | 0.800 | 4.44 | 0.817 | 4.38 | 0.873 | 4.28 |
| 16 | 0.882 | 4.34 | 0.869 | 4.37 | 0.752 | 4.49 | 0.808 | 4.41 | 0.886 | 4.33 | 0.903 | 4.27 |
| 18 | 0.843 | 4.35 | 0.877 | 4.37 | 0.843 | 4.47 | 0.791 | 4.40 | 0.886 | 4.31 | 0.903 | 4.28 |
| 20 | 0.860 | 4.36 | 0.869 | 4.36 | 0.839 | 4.45 | 0.830 | 4.40 | 0.916 | 4.30 | 0.894 | 4.24 |
| 22 | 0.860 | 4.34 | 0.895 | 4.35 | 0.834 | 4.42 | 0.834 | 4.37 | 0.903 | 4.30 | 0.886 | 4.23 |
| 24 | 0.851 | 4.35 | 0.886 | 4.38 | 0.843 | 4.44 | 0.834 | 4.39 | 0.894 | 4.28 | 0.886 | - |
| 26 | 0.774 | - | 0.886 | - | 0.851 | - | 0.817 | - | 0.882 | - | 0.911 | - |
| 28 | 0.800 | - | 0.877 | - | 0.834 | - | 0.826 | - | 0.894 | - | 0.860 | - |

*The S. lactis cultures are designated the same as the butter cultures from which they were isolated.

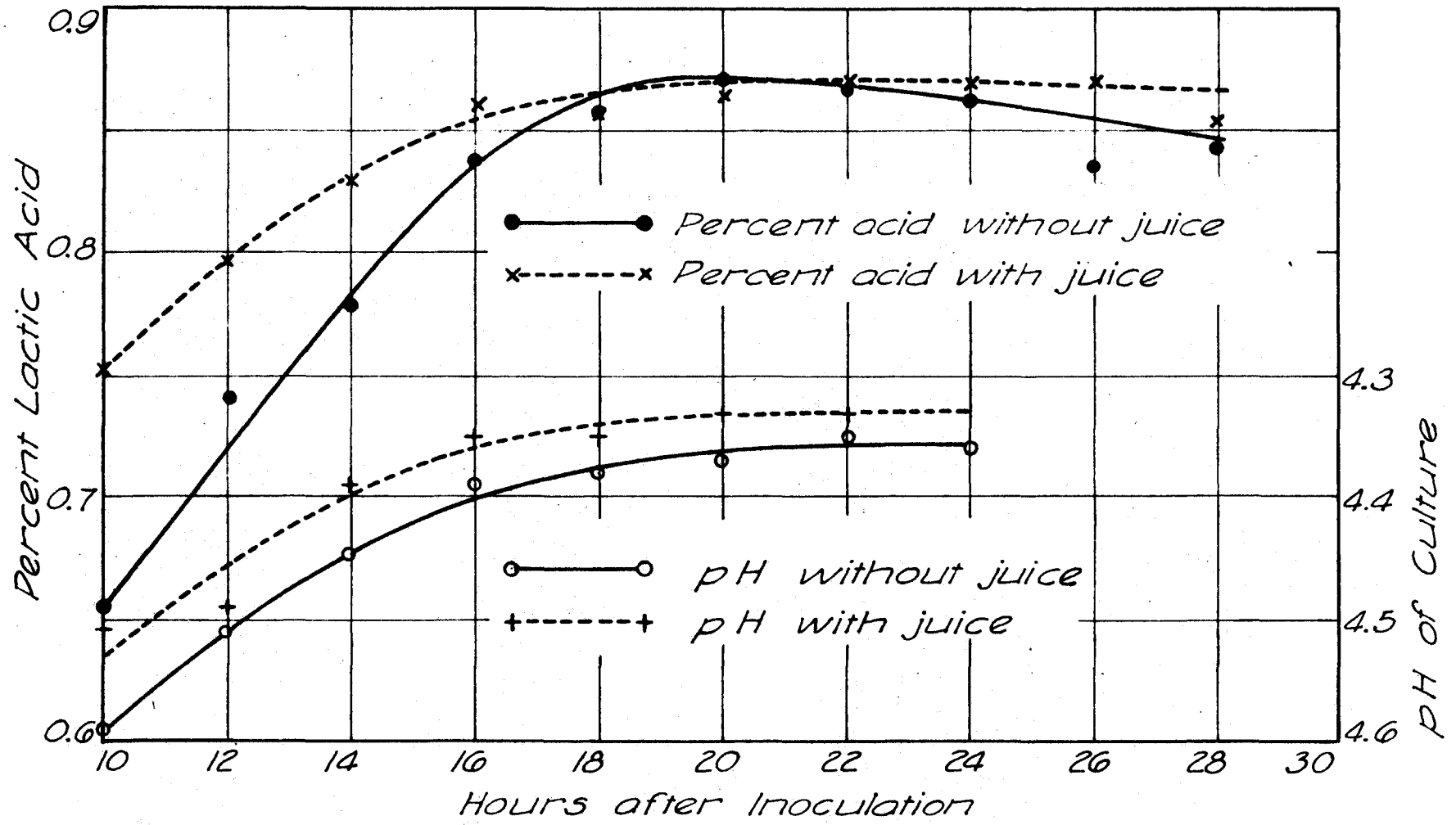


Fig. IV. Effects of tomato juice on acid production in milk. Means of three trials.

controls but remained lower by an amount which appears from the curves to be practically constant.

From the foregoing data it is evident that the titrable acidity produced by S. lactis in a milk culture was not increased by the addition of tomato juice and that the addition of the juice to a milk culture of S. lactis lowered the pH (below the control) by an amount which remained constant throughout the incubation period. When the total acidity (whether arising from the production of lactic acid by S. lactis or from the combination of such lactic acid and the acid of the tomato juice) reached a certain level, the production of further lactic acid by S. lactis was inhibited. This inhibition apparently depended on total acidity rather than on pH, since the peak total acidity of the controls and of the cultures containing tomato juice was identical, while the pH values showed distinct differences. It is possible that the lower pH, which was observed in the cultures containing tomato juice, resulted from the reduced buffer capacity of the milk due to its dilution with tomato juice. In the diluted milk any changes in the concentration of acid would produce greater changes than in the undiluted milk. As the pH of the cultures containing tomato juice did not increase during the fermentation but remained lower than the pH of the controls, it can be assumed that none of the acidic constituents of the tomato juice was attacked by S. lactis.

B. Effects on the Production of Acetylmethylcarbinol plus Diacetyl by a Citric Acid Fermenting Streptococcus in Milk Containing Added Citric Acid

Since the experiment described in the previous section indicates very clearly that tomato juice did not significantly increase the amount of acid produced by S. lactis when the organism was grown in milk, the action of the juice on the production of acetylmethylcarbinol plus diacetyl by a citric acid fermenting organism was investigated.

The citric acid fermenting Streptococcus was inoculated into milk containing 0.15 per cent added citric acid and also into milk containing 5 per cent tomato juice in addition to the citric acid. These two cultures were grown for 20 hours at room temperature and each was subdivided into 8 portions. The acidity of each portion was then adjusted with sterile 20 per cent sulphuric acid so that the pH values of the portions ranged from 5.68 to 3.18. The portions were incubated a further 40 hours at room temperature and the amounts of acetylmethylcarbinol plus diacetyl present and the pH were determined. The results obtained are shown in Table VIII and Figure V.

The cultures to which no tomato juice was added showed the typical results obtained when the pH is adjusted to cover a range of values (16,28). The culture having the highest pH value contained too little acetylmethylcarbinol plus diacetyl to be detected by the analytical method used. As the pH was

TABLE VIII

EFFECTS OF TOMATO JUICE ON ACETYLMETHYLCARBINOL PLUS DIACETYL PRODUCTION IN MILK CONTAINING ADDED CITRIC ACID BY A CITRIC ACID FERMENTING STREPTOCOCCUS

Cultures incubated 20 hours. pH then adjusted with 20 per cent $H_2SO_4$ and cultures incubated for an additional 40 hours.

| Control | | | | 5 per cent juice added | | | |
|---|---|---|---|---|---|---|---|
| pH after adding $H_2SO_4$ | Final pH | Increase in pH during incubation | Mg. of Ni-salt* | pH after adding $H_2SO_4$ | Final pH | Increase in pH during incubation | Mg. of Ni-salt* |
| 5.68 | 5.01 | -.67 | None | 5.66 | 5.58 | .04 | None |
| 5.00 | 4.99 | -.01 | 2.7 | 4.88 | 5.19 | .31 | Trace |
| 4.67 | 5.05 | .32 | 9.5 | 4.67 | 5.02 | .35 | 1.9 |
| 4.48 | 4.91 | .43 | 17.4 | 4.44 | 4.85 | .41 | 18.8 |
| 4.30 | 4.67 | .37 | 33.5 | 4.25 | 4.64 | .39 | 39.4 |
| 4.01 | 4.36 | .35 | 48.4 | 4.03 | 4.43 | .40 | 58.4 |
| 3.61 | 4.25 | .54 | 36.1 | 3.58 | 3.96 | .38 | 79.1 |
| 3.18 | 3.22 | .04 | 13.3 | 3.18 | 3.52 | .34 | 84.0 |

*Mg. of Ni-salt equivalent to acetylmethylcarbinol plus diacetyl per 200g. culture.

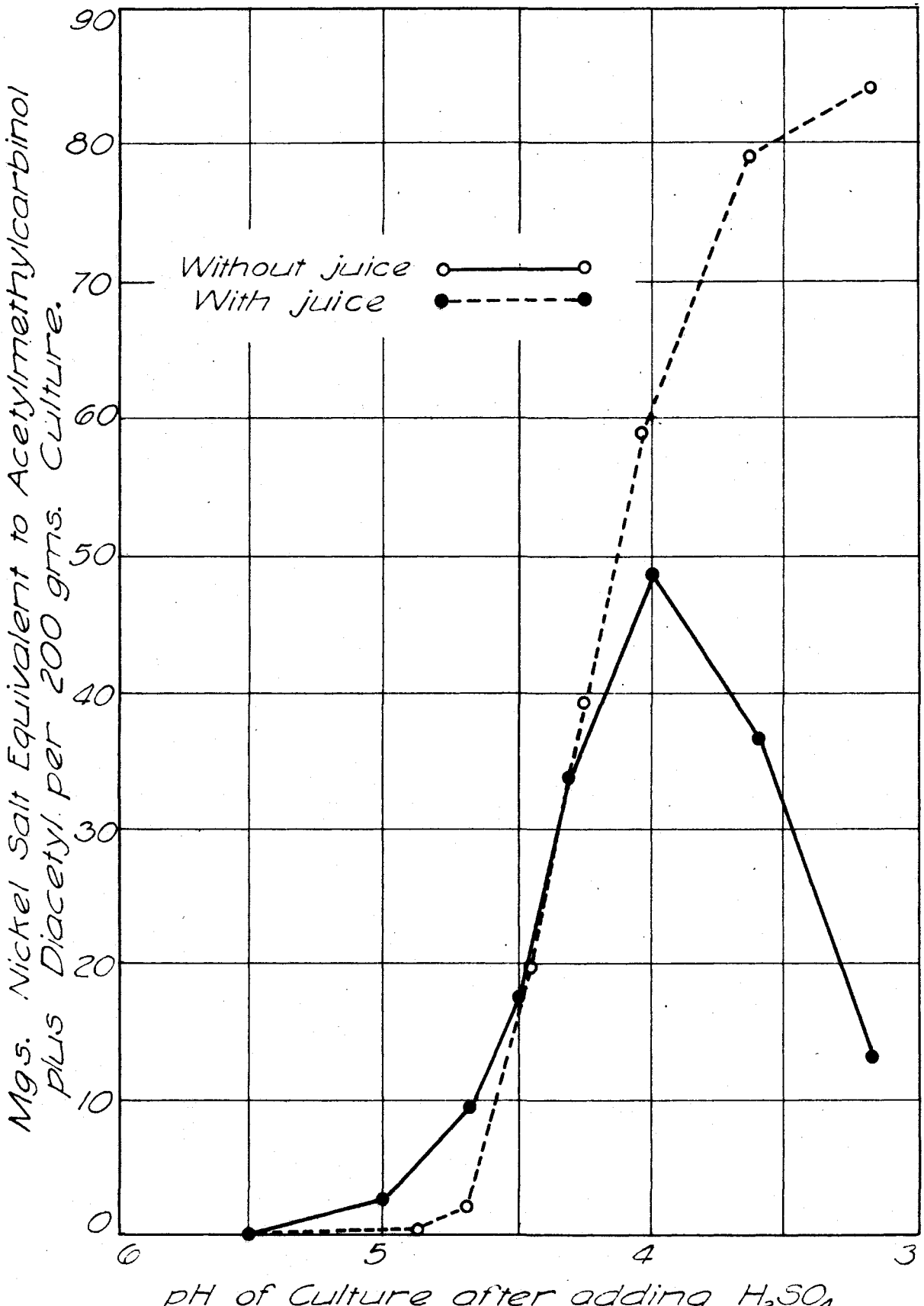


Fig. V. Effects of tomato juice on acetylmethylcarbinol plus diacetyl production in milk containing added citric acid by a citric acid fermenting Streptococcus.

lowered, the yield increased until a peak was reached at a pH value of 4.01, when 48.4 mg. of Ni-salt were obtained. Beyond this point there was a rapid decrease in yield of acetylmethylcarbinol plus diacetyl.

The behavior of the cultures containing tomato was, however, very different. At the higher pH values, the production of acetylmethylcarbinol plus diacetyl was much lower than that of the control, but as the pH decreased, the yield increased very rapidly. The range of pH values covered in the trial did not extend far enough to include the values at which the yield was reduced, but from the shape of the curve, it appears that the peak yield must have occurred at a pH near the lowest used, which was 3.18. This yield was almost twice the maximum obtained when no juice was added. The increase in yield probably was not entirely due to the furnishing of additional substrate (citric acid) by the tomato juice, for if this were the case the maximum yield would be expected at approximately the same pH as in the control. It is difficult to account for the extremely high production which occurred at the low pH values.

It will be observed that in all but two of the determinations a significant increase in pH occurred during the period of incubation. This was due to the decomposition of citric acid by the organisms present. There was no relation between the increase in pH and the amount of acetylmethylcarbinol

plus diacetyl produced, so it appears that the destruction of citric acid is not a measure of the amount of acetylmethylcarbinol plus diacetyl formed.

The data presented above make it evident that tomato juice has a profound effect on the production of acetylmethylcarbinol plus diacetyl by a citric acid fermenting Streptococcus. The greatest increase in yield does not occur, as might be expected, at a pH which is optimal for the production when no juice is present, but at a pH which, under normal conditions, is low enough to depress the yield very markedly.

## Part VII

## Attempts to Concentrate or Dry Tomato Juice

The presence of a large amount of water in tomato juice makes it very difficult to treat with solvents or chemical reagents with a view to dividing it into various fractions, from one of which it might be possible to obtain the material responsible for the acceleratory effect described in Part III.

The first method of concentration which suggested itself was that of direct evaporation on a water bath. During the evaporation, the tomato juice gradually darkened in color and finally a thick dark brown syrup was obtained which, on cooling, formed an amorphous mass. The mass was removed from the evaporating dish with difficulty and was found to be very hygroscopic, so much so that in a day or two the solid mass, which had been kept in a petri dish in a cool chamber, was reduced to a highly viscous fluid. When redissolved in water and diluted to a concentration approximately equivalent to that of the original tomato juice it failed to give increases in the acetylmethylcarbinol plus diacetyl production when added to butter cultures and in certain concentrations actually caused decreases.

Since evaporation to dryness was not successful, a quantity of juice was reduced to half of its original volume by heating on a water bath, but this concentrated juice likewise

failed to increase the yield of acetylmethylcarbinol plus diacetyl. All cultures containing it in various concentrations showed a very marked lowering of the yield. A commercial corporation of wide experience in the production of desiccated biological preparations reported that a dry tomato juice had been prepared. It was so very hygroscopic it could be handled only with extreme difficulty. A sample of the material was examined and the particles coalesced, even during weighing, and became gummy in a short time.

Since these direct methods of drying failed, a method of drying was sought in which some inert carrier could be used to hold the moisture and prevent deliquesence of the dried juice. Various concentrations of agar were used and the solutions of agar in tomato juice were dried in open petri dishes at various temperatures. At 110° C. marked darkening occurred, while at 45° C. darkening occurred unless the dishes were cooled as soon as their contents were dry. When 1 per cent of agar was used it was found to be almost impossible to remove the thin layer of dry juice and agar from the glass, to which it adhered very firmly; this difficulty also occurred when 5 per cent of agar was used, but when the dishes were greased with petroleum jelly it was comparatively easy to remove the dry material. This material was also hygroscopic. On being diluted to the consistency of normal juice, it failed to bring about an increase in yield of acetylmethyl-

carbinol plus diacetyl when added to a butter culture.

The foregoing tests made it seem probable that exposure to the air, at a comparatively high temperature, caused a destruction of the accelerating principle. Other tests have shown that sterilization reduces the activity of tomato juice but when one sample was sterilized twice and then added to butter cultures, it was still able to produce considerable increases in the yield of acetylmethylcarbinol plus diacetyl. Experimental results showed that when 1 per cent of sterilized juice was added to butter cultures the increases in acetylmethylcarbinol plus diacetyl were 49 per cent of those obtained when 1 per cent of raw juice from the same lot was employed. Similarly, additions of 3, 5, 10, 15 and 20 per cent produced increases equivalent to 66, 66, 75, 80 and 72 per cent of the increases in yield obtained when the raw juice was added. Thus, high temperature alone was apparently responsible for only partial destruction of the activity of tomato juice. This suggests that oxidation is responsible for much of the destruction which occurred.

A method was then sought whereby juice could be dried at approximately room temperature. One method which suggested itself was to suspend strips of heavy filter paper approximately 2 inches by 18 inches, so that they dipped into beakers, each of which contained 100 ml. of tomato juice. At first it was hoped that capillary action would be sufficient

to draw the liquid far enough up the strips to ensure rapid drying, but the evaporation proceeded so slowly that copious mold growth occurred before drying was complete. In order to increase the rapidity of drying, an electric fan was set up with two gas burners in front of it, so that a current of warm air passed over the papers which were dipped into the juice as often as they became dry. By this method approximately 100 ml. of tomato juice could be evaporated in about 8 hours. By keeping the papers with the dry tomato juice on them in a desiccator, it was possible to prevent absorption of atmospheric moisture. However, when the paper was macerated in warm distilled water, the reconstituted juice failed to have any marked effect on the production of acetylmethylcarbinol plus diacetyl.

Investigators (4) in England found that it was possible to prepare concentrates of various fruit juices by freezing them slowly and removing the ice as it formed. This method yielded concentrates which had a finer flavor than those prepared by the usual vacuum evaporation. The principle was applied to the concentration of tomato juice in the following way. A liter of the juice was placed in a graduated cylinder and held in an ice cream hardening room having a temperature of about -17° C. As rapidly as ice formed, it was removed by means of a wire net attached to a handle long enough to reach to the bottom of the cylinder. The ice was shaken as free of

juice as possible and transferred to a beaker. When the volume in the cylinder had been reduced by 100 ml., the next ice that formed was placed in a fresh beaker; this general procedure was continued until finally there were 10 beakers each containing ice equivalent to 100 ml. of liquid and representing increasing concentrations of the tomato juice. After about 1 week, the fractions of juice were thawed and their effects on the production of acetylmethylcarbinol plus diacetyl by a butter culture were studied. The yields of these substances when 5 per cent of each of the fractions was added to cultures were as follows:

| Culture | Yield mg. of Ni-salt equivalent to acetylmethyl-carbinol plus di-acetyl per 200g. culture |
|---|---|
| Control - no tomato juice | 34.8 |
| With 5 per cent of fraction 1 | 63.6 |
| With 5 per cent of fraction 2 | 60.4 |
| With 5 per cent of fraction 3 | 66.1 |
| With 5 per cent of fraction 4 | 67.9 |
| With 5 per cent of fraction 5 | 71.6 |
| With 5 per cent of fraction 6 | 71.2 |
| With 5 per cent of fraction 7 | 76.4 |
| With 5 per cent of fraction 8 | 73.6 |
| With 5 per cent of fraction 9 | 75.6 |
| With 5 per cent of fraction 10 | 73.6 |

In general, the yields were increased more by the higher concentrations but there was no close correlation between the yields and the concentrations of juice added to the cultures; even the first fraction was very effective in increasing the

yield of acetylmethylcarbinol plus diacetyl.

If a method were available whereby the ice formed could be freed completely of adhering juice, it seems probable that a greater difference between the various fractions would result. In the method used, much juice was removed along with the ice. The data also indicate that freezing does not destroy the ability of the juice to increase the production of acetylmethylcarbinol plus diacetyl by a butter culture. It was felt however that the freezing method did not concentrate the juice sufficiently to facilitate its study.

An attempt was next made to obtain a stable, relatively non-hygroscopic and active preparation from tomatoes directly. These were washed, the green stem ends were removed, and slices about 0.25 inch in thickness prepared. The slices were placed on waxed paper (to prevent their sticking), covered with cheesecloth (to keep off flies and other insects) and dried in the sun. The time required for drying depends greatly on the temperature, humidity and amount of sunshine, but on a warm,clear, fall day 1 day was usually sufficient to dry each side of the slices. By turning the slices frequently, even drying was brought about. When drying seemed complete, the slices were removed from the wax paper and placed in a container that could be tightly closed. They remained in this for several months without developing mold growth

or appearing moist.

The yield of dried tomato was approximately 4.5 per cent of the weight of the fresh tomatoes, since 20 lbs. of fresh fruit yielded 14.5 ozs. of the dried material. While this type of material is fairly stable, it is slightly hygroscopic and should be kept in a tight vessel at all times to prevent the absorption of moisture from the air.

A quantity of the dried product was finely chopped and 5g. of it were macerated in 100 ml. of warm water for several hours. It was then centrifuged, and the clear juice was decanted. This reconstituted juice was approximately equal in concentration to that from fresh tomatoes. The effect of the juice from the dried tomatoes was compared with the effect of regular tomato juice by adding 5 per cent of each type of juice to cultures prepared in the usual way; control cultures (without juice) were also included. Typical results are as follows: in trial I the juice from the dried tomatoes was used raw, in trial II it was boiled and in trials III and IV it was sterilized.

| Culture | Yield mg. of Ni-salt equivalent to acetylmethylcarbinol plus diacetyl per 200g. culture | | | |
|---|---|---|---|---|
| | Trial I | Trial II | Trial III | Trial IV |
| Control | 54.2 | 59.9 | 59.9 | 9.7 |
| Culture + regular juice | 79.0 | 86.0 | 64.4 | 23.0 |
| Culture + juice from dried tomatoes | 72.1 | 85.4 | 73.9 | 21.5 |

The juice reconstituted from dried tomatoes appeared to be as effective as juice prepared from canned tomatoes in accelerating the production of acetylmethylcarbinol plus diacetyl by butter cultures. Boiling and sterilizing apparently did not affect the potency of the juice.

## Part VIII

## Various Factors Modifying the Effect of Tomato Juice on Production of Acetylmethylcarbinol plus Diacetyl by Butter Cultures

Various factors which might influence the acceleratory effect of tomato juice on the production of acetylmethylcarbinol plus diacetyl by butter cultures were studied in order to develop a procedure for preparing juice so that its activity would not be seriously impaired. Some of these factors have already been discussed but the results will be summarized here so that a complete picture can be obtained.

### A. Effect of Heat

A large batch of tomato juice was usually prepared at a time. To preserve it until needed, it was sterilized.

To study the effect of exposing juice to the sterilizing temperature, 121° C., for 25 minutes, a quantity of juice was prepared from fresh tomatoes. A portion of it was sterilized and then the raw and the sterile juices were added to butter cultures in concentrations ranging from 1 per cent to 20 per cent. A summary of the yields of acetylmethylcarbinol plus diacetyl obtained is given below:

| Culture | Yield mg. of Ni-salt equivalent to acetylmethyl-carbinol plus di-acetyl per 200g. culture | | Yield obtained with sterile juice as per cent of yield with raw juice |
|---|---|---|---|
| | Sterilized juice | Raw juice | |
| Without juice | 20.8 | 20.8 | - |
| With 1 per cent juice | 24.0 | 47.0 | 49 % |
| With 3 per cent juice | 34.4 | 52.6 | 66 % |
| With 10 per cent juice | 45.4 | 60.9 | 75 % |
| With 15 per cent juice | 50.8 | 62.3 | 80 % |
| With 20 per cent juice | 48.6 | 66.8 | 72 % |

From the above it is evident that, on sterilizing, the potency of fresh tomato juice was reduced from 20 to 51 per cent, depending on the concentration used.

When juice was exposed to heat in open vessels, however, the potency was greatly reduced and frequently the heated juice appeared to exert an inhibitory action. In attempts to produce a tomato juice concentrate, one portion of canned tomato juice was evaporated almost to dryness, and another portion was evaporated to about half its bulk, by heating on a water bath. The material which was almost dry was diluted to approximately the concentration of the original juice and added to butter cultures while the partly dried material was added without dilution. The effects of the two materials on the yield of acetylmethylcarbinol plus diacetyl were as follows:

| Yield with no juice added mg. of Ni-salt equivalent to acetylmethyl carbinol plus diacetyl per 200g. culture | Material added to culture | Yield with juice added mg. of Ni-salt equivalent to acetylmethyl-carbinol plus diacetyl per 200g. culture |
|---|---|---|
| 38.5 | 1 per cent reconstituted juice | 26.8 |
| 38.5 | 2 per cent reconstituted juice | 36.9 |
| 72.8 | 5 per cent concentrated juice | 61.8 |
| 62.2 | 10 per cent concentrated juice | 38.8 |
| 72.8 | 10 per cent concentrated juice | 55.2 |
| 62.2 | 20 per cent concentrated juice | 3.7 |
| 72.8 | 20 per cent concentrated juice | 32.5 |
| 62.2 | 40 per cent concentrated juice | 5.8 |
| 72.8 | 40 per cent concentrated juice | 40.9 |

These results show wide variations, yet it is evident that long exposure to a high temperature in an open vessel caused the materials to lose their original acceleratory property and to inhibit, partially, the production of acetylmethylcarbinol plus diacetyl.

An attempt was also made to concentrate juice from canned tomatoes by drying it on strips of filter paper. After the juice had been taken up by these strips they were macerated in warm water and the effect of the reconstituted juice on the production of acetylmethylcarbinol plus diacetyl was determined. A control culture containing no juice yielded 45.9 mg. of Ni-salt, one containing 5 per cent of ordinary sterile juice produced 54.1 mg. and the culture containing the reconstituted juice yielded only 17.5 mg.

To study the effect of exposure of the juice to boiling

temperatures for a short time, a portion of juice from canned tomatoes was boiled for 10 minutes in a beaker and then 5 per cent of this boiled juice and 5 per cent of juice from the same lot which had not been boiled, were added to cultures. The control culture containing no juice yielded 59.2 mg. of Ni-salt, that containing the unboiled juice yielded 86.0, and the boiled juice yielded 86.4 mg.

From the data presented in the preceding paragraphs it appears that exposure to boiling temperatures for a short time did not affect the activity of the juice, and exposure to sterilizing temperatures for 25 minutes reduced the activity only partially, but that exposure in thin layers to air (on filter paper) or heating for a long period in contact with air on a water bath destroyed the activity of the juice and caused it to inhibit the production of acetylmethylcarbinol plus diacetyl to varying degrees.

B. Effect of Freezing

The use of low temperatures to concentrate juice has been discussed in Part VII. It was found that freezing and holding the juice at approximately -17° C. for 1 week did not affect its ability to increase the yield of acetylmethylcarbinol plus diacetyl.

C. Effect of the Salt Content

In Part II various brands of tomato juice were analyzed

for salt and it was noted that the three brands which inhibited the production of acetylmethylcarbinol plus diacetyl the most, were the ones which had the highest salt contents. The lowest salt contents were found in juices prepared from fresh tomatoes and these juices produced relatively large increases in acetylmethylcarbinol plus diacetyl.

D. Effect of Separating the Solid Material from the Juice

The tomato juice throughout these studies consisted of a clear juice separated from solid material by filtering. To determine whether the solid material had any greater effect than the clear juice in increasing the yield of acetylmethylcarbinol plus diacetyl, a quantity of pulp was prepared from fresh ripe tomatoes. One portion was strained before being sterilized and the other was not. On the addition of these two portions to cultures in various concentrations, the following results, which are the means of three trials, were obtained:

| Culture | Yield mg. of Ni-salt equivalent to acetylmethylcarbinol plus diacetyl per 200g. culture | |
|---|---|---|
| | Sterilized juice added | Sterilized pulp added |
| With out addition | 20.8 | 20.8 |
| With 1 per cent addition | 24.0 | 30.3 |
| With 3 per cent addition | 34.4 | 34.9 |
| With 10 per cent addition | 45.4 | 51.3 |
| With 15 per cent addition | 50.8 | 48.4 |
| With 20 per cent addition | 48.6 | 49.8 |

In general, there is no marked difference between sterilized juice and the sterilized pulp from the standpoint of their effect on the production of acetylmethylcarbinol plus diacetyl, indicating that the active principle is present in the juice, rather than in the solid material.

The juice obtained after filtering the pulp was usually quite clear, but during sterilization it became cloudy and on standing a precipitate formed. To determine whether this precipitate had any effect on the yield of acetylmethylcarbinol plus diacetyl a sample of sterilized juice showing considerable precipitate was agitated thoroughly to ensure a uniform distribution of the solid material and then centrifuged for about 3 hours. At the end of this time the solid material was found to be in a mass at the bottom of the tube and the supernatant liquid was very clear.

The solid material obtained was mixed with water. This suspension, the supernatant liquid and a sample of juice which had not been centrifuged were added to cultures and the amounts of acetylmethylcarbinol plus diacetyl produced were determined. The suspension and the supernatant liquid represented an amount of juice equivalent to 5 per cent of the culture, and the juice that was not centrifuged made up 5 per cent of the culture.

The control culture with no tomato juice yielded 80.5 mg.

of Ni-salt, that containing the untreated juice yielded 97.3 mg., that containing the centrifuged juice yielded 92.9 mg. and that containing the solid material yielded 89.7 mg. Thus, centrifuging the juice appeared to reduce its potency only slightly, while the solid precipitate appeared to increase the yield slightly over the control.

On the basis of the influence of the various factors discussed above, on the effect of tomato juice on the yield of acetylmethylcarbinol plus diacetyl in butter cultures, various suggestions can be made regarding the preparation of tomato juice of high potency.

A high salt content may be related to an inhibitory action on the yield, thus if canned tomatoes are to be used as a source of juice, a brand should be chosen which is relatively low in salt. A low salt content, however, did not ensure that the juice will be active, as some brands, having quite low salt contents were found to be quite ineffective in increasing the yield of acetylmethylcarbinol plus diacetyl by butter cultures.

Sterilizing a juice, which was otherwise satisfactory, left it fairly potent, while prolonged exposure to air at high temperatures reduced the potency very greatly. The juice

should therefore be sterilized in screw-capped rather than cotton stoppered bottles, and after removal of the bottles from the autoclave, the caps should be screwed down tightly to prevent the ingress of air as the contents cool.

If critical biological work were being done, it might be advisable to prepare the juice from fresh ripe tomatoes, as this is known to be highly potent and would be free of added salt. To avoid the reduction in potency due to sterilizing, the juice could be filtered through a Chamberland or Berkefeld filter, as tests have shown that the active principle was associated with the juice and not with the solid material, so that the efficiency probably would not be reduced by filtering. The juice could be collected in sterile vessels and frozen until needed, as trials have shown that freezing did not reduce the activity of the juice.

## Part IX

## Effects of Ether and Alcohol Extracts of Tomatoes on the Production of Acetylmethylcarbinol plus Diacetyl by Butter Cultures

When it was definitely established that tomato juice exerted a marked acceleratory effect on the production of acetylmethylcarbinol plus diacetyl by butter cultures, an attempt was made to isolate various fractions which might contain the substance or substances responsible for the increase in the rate of production.

### A. Effects of Ether Extracts

Ether extracts were obtained in three ways. With the first procedure 1000 ml. of tomato juice were shaken with 100 ml. of di-ethyl ether. The mixture was allowed to stand for several days and then it was salted out with sodium chloride. The ether layer was separated from the aqueous one, and evaporated to dryness at room temperature. The residue left after evaporation was shaken well with 100 ml. of water but being of a lipoid nature did not dissolve completely. As this extract from 1000 ml. of juice was redissolved in 100 ml. of water, it should have contained the ether soluble materials in approximately 10 times the concentration in which they were present in the original juice.

The second ether soluble fraction was prepared by extracting strips of filter paper on which tomato juice had been

dried as described in Part VII. The paper was cut into small pieces, placed in an extraction thimble and extracted in a Soxhlet apparatus. The ether was allowed to evaporate at room temperature and the residue was taken up in sufficient water to give the same volume as that of the juice originally used. Accordingly, the suspension of ether soluble material should have been of approximately the same concentration as in the original juice.

When it was found that the dry tomatoes, prepared according to the method described in Part VII, produced a juice which retained its potency, the third ether soluble fraction was obtained by extracting finely chopped dry tomato. In this way a more complete extraction could be obtained than by shaking the juice, as instead of relying on the distribution co-efficient of the ether soluble material to cause it to be more concentrated in the ether layer, the extraction could be continued until no further material was dissolved from the tomatoes. When the ether was evaporated, it was found that, from 5g. of the dry material, about 0.1 ml. of reddish brown, oily material were obtained. This amount of extract was obtained from about 100g. of fresh tomato, as the yield of dry tomato was about 4.5 per cent of the weight of fresh tomatoes. The extract was added to 50 ml. of water, so that the suspension contained the ether soluble material in approximately twice the concentration in which it was

present in the original tomatoes. As has already been mentioned, the ether extract did not dissolve in water. In order to increase its distribution throughout the cultures to which it was added, it was emulsified. The extract from 5g. of dry tomato was added to 50 ml. of a 0.5 per cent agar solution in a bottle containing glass beads. When the water was heated sufficiently to melt the agar and the bottle was shaken vigorously, it was found that the emulsifying effect of the dilute agar solution and the mechanical effect of the glass beads combined to form a fairly stable emulsion of the ether soluble fraction.

The three ether extracts were added to cultures and the production of acetylmethylcarbinol plus diacetyl was determined. The results obtained are summarized in Table IX. From the data given it appears that the ether extract of tomato juice does not influence the yield significantly. When the mean difference of the determinations is calculated, this is found to amount to -.55 mg., indicating that in general the ether extract has a negligible effect on the yield. It is of interest to note, however, that a suspension of the ether extract in agar appeared to produce significant increases in two out of the three trials in which it was used. This seems to indicate that the failure of the ether extract to produce an increase in yield may have been due, in part to the fact that it was not sufficiently distributed throughout

TABLE IX

EFFECTS OF ETHER EXTRACTS OF TOMATOES ON THE PRODUCTION OF ACETYLMETHYLCARBINOL PLUS DIACETYL BY BUTTER CULTURES

| Concentration of material added | Material added to cultures | Mg. of Ni-salt* | | | |
|---|---|---|---|---|---|
| | | Trial I | | Trial II | |
| | | Yield with ether extract added | Increase in yield of Ni-salt over control | Yield with ether extract added | Increase in yield of Ni-salt over control |
| 0.1 % | Ether extract, prepared directly from juice, suspended in water | 83.6 | -0.2 | 60.6 | -4.4 |
| 0.5 % | | 80.6 | -3.2 | 65.3 | +0.3 |
| 1.0 % | | 77.1 | -6.7 | 54.8 | -10.2 |
| 2.0 % | | 78.8 | -5.0 | 54.9 | -10.1 |
| 5.0 % | | 57.0 | +11.1 | 66.7 | -2.6 |
| 10.0 % | | 52.2 | +6.3 | 65.5 | -3.8 |
| 5.0 % | Ether extract of juice dried on paper, suspended in water | 43.8 | -2.1 | 64.8 | -4.5 |
| 10.0 % | | 40.3 | -5.6 | 61.3 | -8.0 |
| 2.5 % | Ether extract of dry tomato suspended in water | 63.8 | +0.5 | 47.6 | +0.5 |
| 2.5 % | Ether extract of dry tomato suspended in 0.5 % agar | 60.8 | +13.7 | 72.6 | +3.7 |
| 5.0 % | | 77.6 | +17.7 | - | - |

*Mg. of Ni-salt equivalent to acetylmethylcarbinol plus diacetyl per 200g. culture.

the culture. Even where the ether extract was emulsified it still appeared that the greater part of the activity remained in the ether insoluble residue. When the addition of the emulsion increased the yield by 13.7 mg., a trial with the residue increased it by 34.3 mg., and when the emulsion increased the yield 17.7 mg., the residue increased it 26.1 mg. This indicates that while the principle may be ether soluble to a limited extent, the residue retains the greater part of the potency.

B. Effect of an Alcohol Extract

To study the effect of the alcohol soluble fraction of tomato juice, 5g. of dry tomato were finely chopped, and shaken with successive 25 ml. portions of 95 per cent alcohol. When the extraction was complete as shown by the alcohol remaining almost colorless, the alcoholic solution was transferred to a flask and the alcohol was distilled off on a water bath. The alcohol soluble fraction was shaken with 100 ml. of distilled water and another portion, prepared in a similar way, was shaken with 0.5 per cent agar solution, in the same way as the third ether extract. These two alcohol extracts were added to butter cultures and the amount of acetylmethylcarbinol plus diacetyl determined. The control culture yielded 68.9 mg. of Ni-salt, the culture to which the alcohol soluble fraction suspended in water was added yielded 77.1 mg. and the culture containing the alcohol

soluble fraction emulsified with agar yielded 81.8 mg. The residue from the alcohol extraction when dissolved in water, produced a yield of 86.3 mg. in the culture to which it was added.

Thus the results obtained here are very similar to those obtained when an ether extract was used. The alcohol soluble fraction produced an increase in the yield but the residue was more effective in increasing the yield.

## Part X

## General Considerations of the Significance of the Citric Acid of Tomato Juice

The evidence already presented indicated that the increase in yield of acetylmethylcarbinol plus diacetyl when tomato juice was added to a butter culture was not due to the citric acid naturally present in the tomato juice (34) but it was not conclusive.

Four lines of reasoning support the idea that the citric acid content of tomato juice can be responsible for only a very small part of the increase observed.

1. As mentioned earlier, preliminary studies (1) revealed that the addition of tomato juice in concentrations as low as 1 per cent produced marked increases in the yield of acetylmethylcarbinol plus diacetyl. It is known that, normally, the acetylmethylcarbinol plus diacetyl present in a butter culture comes from citric acid through the action of the citric acid fermenting streptococci although occasionally atypical strains of S. lactis may produce small quantities of acetylmethylcarbinol (24). The strains of S. lactis in the butter cultures employed were apparently quite normal so that the source of the acetylmethylcarbinol plus diacetyl presumably was citric acid. In the cultures containing no tomato juice the acetylmethylcarbinol plus diacetyl came from the citric acid naturally present in the milk and from that

added at the time of inoculation. If the increase in yield resulting from the addition of tomato juice were due only to the presence of citric acid in the juice it must have contained enough acid to account for all of increased yield.

If it be assumed that the addition of tomato juice to a butter culture did not increase the proportion of acetylmethylcarbinol plus diacetyl to other substances formed from citric acid, the amount of acid necessary can be calculated, as the following example indicates.

The citric acid content of milk from individual cows in Iowa was found to vary from 0.076 per cent to 0.300 per cent and average 0.18 per cent (34). An additional 0.15 per cent of citric acid was added to the milk at the time of inoculation. It is, therefore, assumed that the citric acid content of the culture was approximately 0.33 per cent.

The average yield of six cultures containing 1 per cent of juice was 26.1 mg. Ni-salt equivalent to acetylmethylcarbinol plus diacetyl per 200g. culture, while the yields of the cultures without juice averaged 19.15 mg. Ni-salt. Thus the addition of 1 per cent of tomato juice increased the yield by 6.95 mg.

19.15 mg. Ni-salt were produced from 0.33 per cent citric acid

$\therefore$ 26.1 mg. Ni-salt were produced from $\left(\frac{0.33}{1} \times \frac{26.1}{19.15}\right)$ per cent citric acid. $= 0.4484$ per cent.

Thus it would require the difference between 0.4484 per cent citric acid and that originally present in the culture to produce the increase observed in the yield of acetylmethylcarbinol plus diacetyl. This is, the addition of 1 per cent of tomato juice must have increased the citric acid content by 0.1184 per cent, or the 1 per cent of juice added must have contained 11.84 per cent of citric acid. Similar calculations show that if all the effect were to have come from citric acid present in the added juice then, when 3 per cent was added the juice would have to contain 5.12 per cent of citric acid, and when 5 per cent was added 6.77 per cent would have been necessary.

Analyses (34) of tomato juice show that it contains, on the average, 0.09 per cent free citric acid and an additional 0.06 per cent in the form of insoluble citrate, or a total citric acid content of approximately 0.15 per cent. Therefore, the tomato juice added to cultures, could not have contained sufficient citric acid to bring about the increases observed in the yields of cultures to which it was added. It seems probable therefore that the addition of tomato juice to a culture alters the conditions under which the citric acid already present in the culture is fermented.

2. If the increases in yield, observed when tomato juice is added to butter culture, were only due to the presence

of citric acid in the tomato juice, the increases observed should bear a direct relationship to the amount of juice added, assuming that the effect of the juice on the buffer capacity is relatively unimportant. Typical mean results from a series of determinations are presented in the following summary:

| Per cent of tomato juice added to culture | Increase in yield mg. Ni-salt equivalent to acetylmethylcarbinol plus diacetyl per 200g. culture | Mg. increase in yield produced by each per cent of juice added |
|---|---|---|
| 1 % | 6.6 mg. | 6.6 mg. |
| 3 % | 12.8 mg. | 4.3 mg. |
| 5 % | 21.0 mg. | 4.2 mg. |
| 10 % | 29.3 mg. | 2.9 mg. |
| 15 % | 32.4 mg. | 2.2 mg. |
| 20 % | 25.6 mg. | 1.3 mg. |
| 30 % | 11.1 mg. | 0.37 mg. |

3. According to the data reported in Part III, tomato juice accelerated the production of acetylmethylcarbinol plus diacetyl in a butter culture, but did not significantly increase the maximum yield. During the early stages of the fermentation the production of acetylmethylcarbinol plus diacetyl in the cultures containing tomato juice was accelerated and thus when analyses were made at this time, it appeared that the yields were increased over those of the controls, but if sufficient time was allowed to elapse, both cultures yielded about the same amounts of acetylmethylcarbinol plus diacetyl. This indicated that approximately the same amounts

of citric acid were fermented in both the cultures containing tomato juice and in the controls, but in the former it was fermented more rapidly.

4. As has been mentioned in Part VI, 5 per cent of tomato juice was added to cultures of a citric acid fermenting Streptococcus, containing added citric acid, and the pH values were lowered to various levels by the use of sulphuric acid. It was found that at lower pH values the cultures containing tomato juice yielded larger amounts of acetylmethylcarbinol plus diacetyl than the controls, but the greatest increase in yield was not obtained, as might have been expected, at the same pH value as that of the culture without juice, which produced the largest yield of acetylmethylcarbinol plus diacetyl but at a pH sufficiently low to depress the yield of acetylmethylcarbinol plus diacetyl in the control considerably. It appears thus, that the addition of tomato juice does not cause more citric acid to be fermented under the same conditions as in the controls, but alters the conditions under which citric acid is fermented.

The foregoing data and discussion indicate citric acid was not present in tomato juice in sufficient amounts to account for the increase observed in the yields, and the increases were not proportional to the amounts of juice added,

thus the juice did not furnish additional substrate. This is confirmed by the fact that tomato juice influenced the rate of production, but not the maximum yield of acetylmethylcarbinol plus diacetyl, indicating that substantially the same amounts of citric acid were fermented in both cultures.

In pure culture tomato juice caused a citric acid fermenting Streptococcus to produce a very high yield of acetylmethylcarbinol plus diacetyl at a pH much lower than that at which the peak production of the control culture occurred, indicating that tomato juice did not cause more citric acid to be fermented under the same conditions as when it was absent, but altered the conditions under which the fermentation took place.

# CONCLUSIONS

When 5 per cent of juice from red and yellow fresh ripe tomatoes and from two brands of canned tomatoes was added to a butter culture, considerable increases in the yields of acetylmethylcarbinol plus diacetyl were produced, and no significant differences could be observed between the effects of these four types of juice. Juice from three brands of canned tomatoes produced less marked increases, one of which was not significant, while juice from four brands caused decreases in yield, three of which were significant and approximately the same in magnitude.

When 10 per cent of juice was added to butter cultures, the juices from red and yellow tomatoes were most effective in increasing the yield of acetylmethylcarbinol plus diacetyl, but 10 per cent of them did not give greater increases than 5 per cent. Only one brand of canned tomatoes caused a significantly greater increase in the higher concentration than in the lower. The juices from the fresh tomatoes and one of the brands of canned tomatoes appeared to be equally potent in the two concentrations while juices from the remaining seven brands was less effective in the higher concentrations.

There was no relationship between specific gravity, pH and color after sterilization of samples of tomato juice and

their effects on the yields of acetylmethylcarbinol plus diacetyl by a butter culture. The three brands of canned tomatoes which decreased the yield most had the highest contents of sodium chloride. Tomato juice intended for use in media should be assayed against juice from fresh ripe tomatoes in order to be sure that it will not inhibit the growth or activity of organisms.

The addition of tomato juice to butter cultures accelerated the production of acetylmethylcarbinol plus diacetyl very considerably, but the final amounts of these substances produced were not significantly increased. It appears that from the standpoint of production of acetylmethylcarbinol plus diacetyl, the effect of tomato juice on butter cultures is definitely acceleratory rather than stimulatory.

Cultures differed markedly in their production of acetylmethylcarbinol plus diacetyl and in their response to the presence of tomato juice. Differences occurred between two propagations of the same culture which frequently were quite as great as those between two cultures inoculated simultaneously into milk from the same lot. The treatment of tomato juice with calcium carbonate increased the pH very considerably and various concentrations of neutralized and untreated juices were added to butter cultures containing citric acid and the effects compared. All concentrations of either juice produced increases in the yield, but when 40 per cent was

added, the yield was lower than that obtained by adding 20 per cent. In concentrations up to 20 per cent, the neutralized juice caused greater increases than the untreated juice, but in the higher concentrations the untreated juice was more effective. In concentrations up to 5 per cent, neutralized juice produced the greater increases for each 1 per cent of juice added. The increases for each 1 per cent of juice added were rather uniform in concentrations up to 5 per cent with both types of juice.

In cultures containing added sodium citrate all concentrations of both types of juices increased the yield of acetylmethylcarbinol plus diacetyl but the yields, in general, were lower than those obtained when citric acid was used. The addition of 40 per cent of untreated juice produced the greatest increases in yield. Although the yield of the culture containing 40 per cent of neutralized juice was less than that of the culture containing 20 per cent, the reduction was not as marked as in the series in which citric acid was used. In concentrations up to 3 per cent, neutralized juice produced the greater increases, but above this concentration the untreated juice produced the greater increases.

Tomato juice continued to increase the yield of acetylmethylcarbinol plus diacetyl by a butter culture after it was neutralized.

Attempts were made to precipitate completely the citric

acid of tomato juice as the calcium or barium salt, but these were not successful.

The addition of tomato juice to a pure culture of S. lactis did not increase the total acidity produced but the pH values of the cultures containing tomato juice were lower than those of the cultures without juice by an amount which appeared to remain constant throughout the incubation period. When tomato juice was added to a pure culture of a citric acid fermenting Streptococcus and the pH reduced with sulphuric acid, the yield of acetylmethylcarbinol plus diacetyl was greatly increased, and the greatest increase occurred at a pH which was so low that, in the absence of tomato juice, the yield was greatly decreased.

A juice reconstituted from dried tomatoes appeared to be as effective as juice from canned tomatoes in accelerating the yield of acetylmethylcarbinol plus diacetyl by butter cultures. Boiling and sterilizing apparently did not greatly decrease the potency of this reconstituted juice.

To produce a juice of high potency, a brand of juice low in salt should be chosen and assayed against juice from fresh tomatoes to determine its effectiveness. Care should be taken to avoid excessive exposure to air during processing. For critical work, it is suggested that juice from fresh tomatoes be filtered through a bacteria-proof filter and held frozen until needed.

Ether extracts of tomato juice did not significantly affect the production of acetylmethylcarbinol plus diacetyl by butter cultures and the residue after extraction retained much of its potency. An alcohol extract caused a slight increase in yield but the residue was more effective.

Various considerations show that tomato juice could not contain sufficient citric acid to account for the increases observed when it was added to butter cultures.

ACKNOWLEDGMENTS

The author wishes to express his deep indebtedness to Mrs. A. C. Abbott who made it possible for this study to be carried out. To Dr. B. W. Hammer, who directed the research, for his unfailing counsel and guidance. To Dr. H. C. Olson for assistance in the preparation of the manuscript. To Miss L. van der Lingen who prepared the dry tomatoes, and to members of the Dairy Industry Section of the Iowa Agricultural Experiment Station who propagated the cultures used.

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