

## STUDIES OF HEATED MILK. II. ACETOL AND RELATED COMPOUNDS<sup>1</sup>

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It has been reported previously that the heating of skim milk evidences the formation of carbonyl compounds, and further, that these compounds can be removed from the milk for further study by such procedures as steam distillation or ether extraction (11). Since these compounds are heat generated in the milk, and, as such, represent end products of the chemical changes taking place, it was considered expedient to accomplish their identification insofar as possible.

### EXPERIMENTAL

*Acetol.* Forty pounds of condensed skim milk (29 per cent total solids) were autoclaved in a sealed, stainless steel can at 122° C. for 3 hr. and then cooled to room temperature. The contents of the can were filtered, the curd discarded and the brown whey-filtrate retained for steam distillation. Steam distillation was accomplished using conventional apparatus, mineral oil being employed as an anti-foaming agent. The distillate was collected for a period of 45 min. after which time the distillate was transferred to a separatory funnel and extracted five times with an equal volume of ethyl ether. The ether layers were combined, set aside for other investigations and attention given to the extracted distillate. It was found to give the following reactions: an orange precipitate with 2,4-dinitrophenylhydrazine reagent, iodoform with iodine-potassium iodide reagent and a brownish-red color when submitted to the nitroprusside test, this color becoming a stable greenish-blue upon acidification of the reaction mixture.

Sufficient of the 2,4-dinitrophenylhydrazone (2,4-DNPH) for purification and recrystallization was prepared by adding 0.5 g. of the reagent in 10 ml. of concentrated H<sub>2</sub>SO<sub>4</sub> to 750 ml. of extracted distillate. After 4 hr. the precipitated reaction product was recovered by filtration. It was found to be slightly soluble in alcohol and to give a purple color when treated with dilute alcoholic sodium hydroxide. Strain (13), among others, has indicated that the bis 2,4-DNPH's of glyoxal and diacetyl exhibit a dark blue color on treatment with alcoholic alkali. The product was recrystallized from nitrobenzene and after two such treatments no melting point increase could be effected, the final melting point being 296–297° C. with decomposition.

A search of the literature revealed methylglyoxal as forming a 2,4-DNPH of this melting point, the reported values being 296–297, 297, 298 (2) and 299–300 (7). The carbon, hydrogen and nitrogen analyses of the derivative from

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extracted distillate were observed to compare well with those of the bis 2,4-DNPH of methylglyoxal: Calculated for  $C_{15}H_{12}O_8N_8$ , carbon 41.67 per cent, hydrogen 2.80 per cent and nitrogen 25.92 per cent; found, carbon 41.82 per cent, hydrogen 3.21 per cent, nitrogen 25.67 per cent.

The above data, together with the qualitative reactions given by the extracted distillate, strongly suggested that acetol or methylglyoxal might be the compound in question. Both of these compounds give the same 2,4-DNPH and similar results with many qualitative tests (12). The authentic 2,4-DNPH of methylglyoxal was made and observed to melt at 296–297° C., both alone and when intimately mixed with the derivative prepared from the heated milk distillate.

With the identity of the derivative established, it remained to ascertain whether the parent compound was acetol or methylglyoxal. Both the steam distillate of heated milk and the ethyl ether extractable material from heated milk gave positive results in the test for acetol developed by Baudisch and Deuel (1). This test depends upon the reaction of acetol with *o*-aminobenzaldehyde to give 3-hydroxyquinaldine, which melts at 265° C. and gives a brilliant blue fluorescence in dilute aqueous solution when exposed to ultraviolet light. This reaction is not given by methylglyoxal (1). In conducting the tests for acetol in heated milk, the 3-hydroxyquinaldine was isolated and identified by melting point, as well as by its blue fluorescence.

*Acetaldehyde.* For the most part, the carbonyl compounds of heated milk appear to be present in quantities too small to permit direct study. However, the conversion of these compounds into their 2,4-DNPH's afforded a practical approach to the problem, providing some suitable method could be developed for separating and purifying the mixed derivatives. With this aim in mind, chromatographic separation of the derivatives prepared from the ether extract of heated milk was attempted. These experiments were largely unsuccessful. However, it seems salient, in view of recent findings (10), to report the isolation of the 2,4-DNPH of acetaldehyde in one instance. The pure crystalline derivative was found to melt at 166–167° C. and to give elemental analyses which compared very well with those calculated for the 2,4-DNPH of acetaldehyde, melting point, 168° C. (9). The mixed melting point of the unknown and an authentic derivative showed no depression (166–167° C.).

*Acetic acid.* During certain phases of this investigation, the presence of acetic acid in ethyl ether extract residues from heated milk was fairly obvious by odor alone. Since the presence of this compound in heated milk does not appear to have been reported previously, a concerted effort was made to confirm the fact. Acetic acid was isolated and identified both from the steam distillate and the ether extract of heated milk. For the sake of brevity, only an account of the steam distillate isolation will be presented.

Concentration of the acids present in 2 l. of steam distillate, collected from 50 lb. of autoclaved skim milk (29 per cent total solids), prepared as previously described, was effected as follows: The pH of the distillate was adjusted to the phenolphthalein end point with sodium carbonate, after which treatment the distillate was extracted four times with equal volumes of ethyl ether. The ex-

tracted distillate was concentrated under vacuum to a volume of 75 to 100 ml. This concentrate was acidified to liberate the acids and then extracted five times with 100-ml. volumes of ethyl ether. The combined ether layers were dried and the solvent removed by evaporation in a water bath at 50° C. The pungently acidic residue (3 g.) was distilled and yielded three fractions boiling as follows: 99.5–100, 100–110 and 110–115° C. Distillation was stopped at 115° C., since it had been noted previously that the temperature rose very rapidly thereafter and that decomposition of the residue occurred. The two lower-boiling fractions were observed, by qualitative tests, to contain appreciable quantities of formic acid. The fraction boiling 110–115° C., of about 0.5 g. in weight, had a strong, distinct odor of acetic acid and contained no formic acid, as evidenced by a very slow reaction with  $\text{KMnO}_4$  reagent. A *p*-nitrobenzyl ester derivative, melting point 77–78° C., was prepared from this fraction. This derivative showed no depression in melting point on admixture with *p*-nitrobenzylacetate, the accepted melting point for which is 78° C. (8). In additional experiments, the presence of acetic acid was further confirmed by observation of refractive index,  $n_D^{25}$  1.372; the preparation of the *p*-bromphenacyl ester, melting point 84–85° C. (8) and conversion of the acetic acid to ethyl acetate.

*Control experiments.* Ten pounds of condensed skim milk, processed in the same manner as the condensed milks used for autoclaving, were steam distilled for a period of 45 min. Periodic tests on the distillate indicated an absence of carbonyl compounds during the first 25 min., after which time a very slight positive reaction with 2,4-dinitrophenylhydrazine reagent could be noted. The absence of these compounds at the start of the treatment would appear to be the critical point. With the autoclaved milks, the first 5 to 10 min. of distillation gave the greatest yield of carbonyl compounds, after which time they were continuously evolved at a lower rate. The distillate from the non-autoclaved milk exhibited no distinct acidity. Thus, the chemical differences in the steam distillates of autoclaved and non-autoclaved condensed skim milks were amply demonstrated for the purposes of this investigation.

#### DISCUSSION

The results of this investigation have established acetol as one of the compounds produced in milk by heat. However, the possibility that methylglyoxal was copresent has not been precluded by this study.

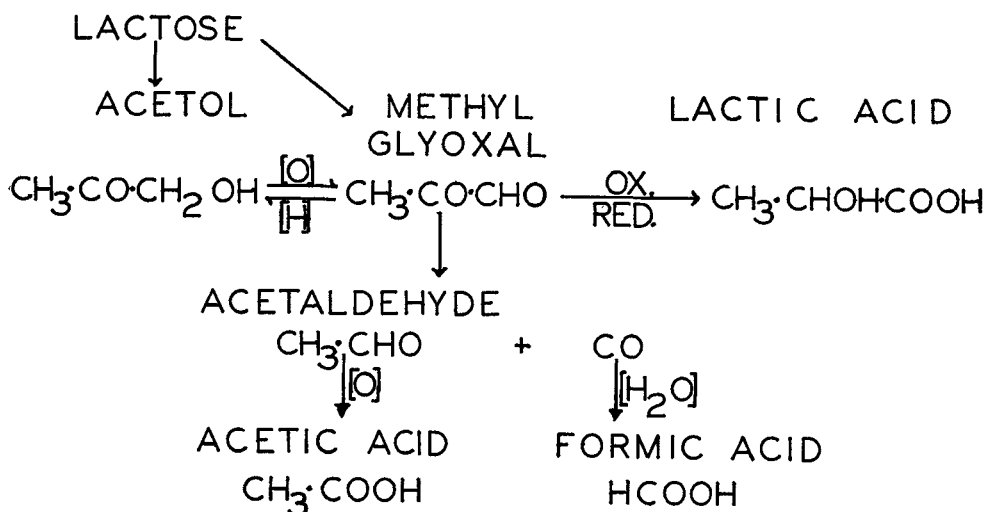
From earlier literature in the field of carbohydrate chemistry (1, 4), it might have been anticipated that acetol or methylglyoxal is formed in milk as a result of heating. Merely heating aqueous sugar solutions has been reported to produce small quantities of the above compounds (3). However, the matter of distinguishing between acetol and methylglyoxal when present at great dilution poses a difficult problem. This subject has been reviewed thoroughly and greatly clarified in a recent paper by Sattler and Zerban (12).

Although data in the literature concerning the physical and chemical properties of methylglyoxal are scarce, observations concerning the acetol-methylglyoxal relationship in these experiments indicated that the latter is present in traces,

if at all. Methylglyoxal is reported to commence boiling at 72° C. (7). No such compound was detected in this study. On the other hand, the ether extract fraction giving a positive Baudisch and Deuel test for acetol, was observed to be relatively stable and to boil in the vicinity of 140° C. These properties are in keeping with those of acetol. Sattler and Zerban (12) have observed a ratio of roughly 500 parts of acetol to one part of methylglyoxal in their study of this matter. It seems unlikely that methylglyoxal, an unstable, highly reactive compound, would exist in free form, in heated milk (122° C.) for any appreciable length of time.

The mechanism of acetol and methylglyoxal formation from sugars has been reviewed (3, 12). While the interrelationship of the compounds reported in this paper is a matter of conjecture, the following scheme is presented as accounting in a logical manner for the formation, in part at least, of certain of these and other compounds known to be produced in autoclaved milk.

Gould (5, 6) has demonstrated the presence of lactic and formic acids in heated milk. The present paper gives consideration to the other compounds concerned in the above scheme.



The presence of acetaldehyde in heated milk has not been firmly established in this investigation, since it was isolated only once in the form of its 2,4-DNPH derivative. However, in view of recent findings by Mohammad *et al.* (10), it seems worthy to note the presumptive presence of this compound in heated milk. The aforementioned group has observed that the rate of browning of protein-acetaldehyde systems is about 35 times as fast as that observed with protein-glucose systems under comparable conditions.

SUMMARY AND CONCLUSIONS

This study has demonstrated the presence of acetol and acetic acid in autoclaved condensed skim milk. The results of adequate control experiments preclude that these compounds are present in significant amounts in unheated

milk. Presumptive but not conclusive evidence that acetaldehyde is formed during the heating of milk also was obtained.

The relationship of acetol to methylglyoxal and certain other compounds known to be present in heated milk is discussed.

A wide variety of carbonyl compounds were observed to be formed during the prolonged heat treatment of milk. These compounds, for the most part, have not yet been identified.

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#### REFERENCES

- (1) BAUDISCH, O., AND DEUEL, N. J. Detection of Thymine in the Presence of Sugar. *J. Am. Chem. Soc.*, **44**: 1581-1585. 1922.
- (2) BEILSTEIN'S HANDBUCH DER ORGANISCHEN CHEMIE. Berlin. Vol. 17: 819. 1941.
- (3) EVANS, W. L. Some Less Familiar Aspects of Carbohydrate Chemistry. *Chem. Revs.*, **31**: 537-560. 1941.
- (4) EVANS, W. L., EDGAR, R. H., AND HOFF, G. P. The Mechanism of Carbohydrate Oxidation. IV. The Action of KOH on d-Glucose and d-Galactose. *J. Am. Chem. Soc.*, **48**: 2265-2277. 1926.
- (5) GOULD, I. A. Lactic Acid in Dairy Products. III. The Effect of Heat on Total Acid and Lactic Acid Production and on Lactose Destruction. *J. Dairy Sci.*, **28**: 367-377. 1945.
- (6) GOULD, I. A. The Formation of Volatile Acids in Milk by High Temperature Treatment. *J. Dairy Sci.*, **28**: 379-386. 1945.
- (7) HEILBRON, I. *Dictionary of Organic Compounds*. Oxford University Press, New York. Vol. 3, p. 564. 1946.
- (8) HUNTRESS, E. H., AND MULLIKEN, S. P. *Identification of Pure Organic Compounds. Order I*. John Wiley and Sons, Inc., New York. p. 180. 1941.
- (9) MCILVAIN, S. M. *The Characterization of Organic Compounds*. The MacMillan Co., New York. p. 200. 1947.
- (10) MOHAMMAD, A., OLCOTT, H. S., AND FRAENKEL-CONRAT, H. The Reaction of Proteins with Acetaldehyde. *Arch. Biochem.*, **24** (2): 270-280. 1949.
- (11) PATTON, S., AND KEENEY, D. G. Isolation of Minor Organic Substances from Heated Milk. *J. Dairy Sci.*, **32**: 698. 1949.
- (12) SATTLER, L., AND ZERBAN, F. W. Unfermentable Reducing Substances in Molasses. Volatile Decomposition Products of Sugars and their Role in Melanoidin Formation. *Ind. Eng. Chem.*, **41**: 1401-1407. 1949.
- (13) STRAIN, H. H. 3-nitrobenzohydrazones, 2,4-dinitrophenylhydrazones and the Separation of Hydrazones by Adsorption. *J. Am. Chem. Soc.*, **57**: 758-761. 1935.