termed hydrocarbo bases. The behavior of mercury dialkyls and diaryls towards hydrolyzing agents, especially glacial acetic acid, lead to the conclusion that these compounds behave as electromers and that the two radicals seem to be one positive and the other negative, R+–Hg+–R. The products obtained by the action of acetic acid, *viz.*, metallic mercury, a hydrocarbon, RH, and an alcohol ROH, seem to substantiate this claim. Similarly tetralkyl lead compounds react as if 3 of the radicals were negative and the fourth radical positive, $(R^-)_3 \overset{+}{\underset{+}{\ddagger}} Pb- + R-$.

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[CONTRIBUTION FROM KENT CHEMICAL LABORATORY OF THE UNIVERSITY OF CHICAGO.]

AN ATTEMPT TO FILTER THE ENZYMES OF MILK.[1]

BY JEAN PICCARD AND MARY RISING.

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Milk, cow's as well as human, contains certain enzymes of unknown origin and chemical constitution.[2] Since nature provides these compounds in natural foods for the young it is safe to assume that they have their important uses, although unknown to us.

If this conclusion is warranted, the value of a sterilized milk with active enzymes is apparent. For artificial feeding of infants boiled, "pasteurized" or "certified" milk is used. Boiled milk contains no enzymes, these being destroyed at a temperature of 72–80°. Milk, pasteurized for 30 minutes at 60°, gives a positive test for enzymes; but the milk is not filtered and suspended dirt remains in it, and there is no certainty that all the dangerous germs and their spores are killed at the relatively low temperature. Certified milk is obtained by methods in which special precautions are observed, but such milk contains, nevertheless, bacteria which multiply rapidly. Milk sterilized by filtration would be free from germs and suspended impurities and any change of the enzyme would be avoided.

With this ultimate aim the experiments described in this paper were undertaken for the purpose of discovering a method of filtering milk enzymes. Repeated attempts by us to accomplish this have met with failure, and no filter has been found with pores large enough to allow the passage through them of the colloidal enzyme body, without at the same time allowing the passage of bacteria. The results obtained and observations made should, however, prove of value to these interested in this problem.

Certified milk about 12 hours old was used in our experiments. We

[1] The work reported on in this paper will form part of the dissertation of Miss Rising for the degree of Doctor of Philosophy at the University of Chicago.

[2] J. König, "Chemie der menschlichen Nahrungs und Genussmittel.," 3, pp. 238–9.

tested only for the Shardinger enzyme.[1] The reaction for this in milk is discussed by Wieland[2] in his paper on catalyzing agents. This reaction, upon which all of our experiments and conclusions depend, is as follows: If to milk methylene blue and salicylic aldehyde are added then in presence of the Schardinger enzyme, which is a catalytic agent, the salicylic aldehyde will reduce and decolorize the methylene blue and be itself oxidized to salicylic acid. When the enzyme is destroyed by boiling or other means, the oxidation reduction reaction takes place so slowly, that it is not perceptible at all.

The application of this test was made by us under certain conditions which are to be considered standard for all experiments described, unless otherwise specified. To a test-tube containing 5 cc. of milk, or milk serum, were added a drop of salicylic aldehyde and 3 drops of methylene blue solution (concentration 1 g. per liter of water). The size of the drops was kept fairly constant by the use of a buret. The mixture was thoroughly shaken and the test-tube placed at once in a water bath kept at 65°, and the time of reduction of the dye was noted. From the deep blue color, the change proceeds through green to a faint yellow. The comparative times of reduction were used as standards for tests and conclusions.

The first plan of procedure included 4 steps: (1) the removal of the fat globules from the milk with the object of thinning the liquid as much as possible, thus making it suitable for convenient filtration; (2) the sterilizing of the milk solution by filtration through a germ-proof filter; (3) the sterilization of the fat, previously removed, by heat; (4) the subsequent mixing of the two sterilized fractions. This plan for sterilization should prove satisfactory, provided the enzyme content stays in the milk solution, instead of separating out with the fat.

The use of the word "milk" in what follows refers to whole milk. "Milk solution" means milk from which fat has been removed, and "milk serum" the filtrate in the filtration experiments.

Centrifugation was tried as a means of removing the fat. Milk was rotated in an electric centrifuge for 5 minutes, and the milk solution was then removed from the thick and partially hardened mass of fat above it by means of a pipet forced through the cream layer into the milk. This method of removing the milk solution from the centrifuge tube was not good, as particles of cream adhered to the pipet and became mixed with the solution. Later we used the method of Van Slyke[3] for the complete

[1] F. Schardinger, *Z. Unters, Nahr. Genussm.*, 5, 1113 (1902); *Chem. Zentr.*, 1, 98 (1903); *Chem. Ztg.*, 28, 704 (1904); *Chim.*, 2, 737 (1904).

[2] H. Wieland, "Über den Mechanismus der Oxidationsvorgänge, *Ber.*, 46, 3339–40 (1913).

[3] L. L. Van Slyke, and A. W. Bosworth, "Condition of Casein and Salts in Milk," *J. Biol. Chem.*, 20, 135 (1915).

separation of fat from the milk solution, and obtained better results. To a liter of milk, 50 cc. of chloroform was added. Chloroform was found not to affect the activity of the Schardinger enzyme.[1] After being thoroughly mixed with the chloroform, the milk was centrifugated as described above. The chloroform layer containing the dissolved fat formed a hard mass at the bottom of the tube, from which the milk solutions was decanted.

Whole milk gave a reduction time of $7^1/_4 \pm {}^1/_4$ minutes; centrifugated milk $5^1/_4 \pm {}^1/_4$.

The fact that the enzymes act more readily when the fat is not present cannot be definitely accounted for. Possibly the fat, when present, absorbs the salicylic aldehyde, and by so doing renders it less active as a reducing agent for the dye.

The separated cream, diluted with water, gave also an enzyme test, so it is certain that not all the enzymes of the milk remain in the milk solution after centrifugation. This would mean a loss of a small part of the enzyme content of the milk in the heat sterilization of the cream.

The first filter selected for trial, as absolutely germ-proof, was the Pasteur-Chamberland filter of porous clay and asbestos. The milk solution was filtered through it under pressure obtained by an apparatus illustrated in the accompanying diagram.

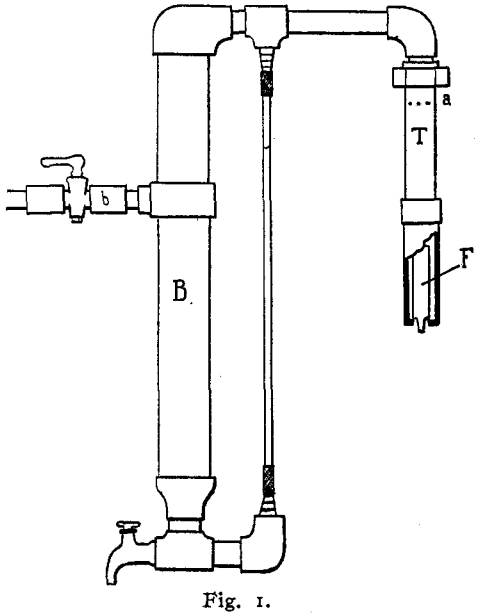

Fig. 1.

The iron tube T, above the filter F, was filled with milk solution to a level indicated by the dotted line *a*. The air in the remainder of the apparatus was compressed by allowing water to flow from the water main through the tube *b*, into the larger tube B. The pressure obtained was from 2–3 atmospheres, but even with such a pressure the filtration was slow. The filter became quickly clogged, and was found to be coated with a slimy deposit of casein.

[1] Chloroform had no effect upon the enzyme content, when used in the proportions indicated in preparing for centrifugation, *i. e.*, 50 cc. per liter. Milk treated with twice and with 4 times that amount, and allowed to stand for 4 hours with the chloroform, gave the enzyme test in the same length of time as fresh, untreated milk gave it.

The milk serum obtained by filtration was of a greenish color and slightly opalescent. An analysis of milk filtered under the same conditions has been published by Van Slyke.[1] He found that the content of the serum is not constant until enough of the milk solution has passed through the filter to saturate it thoroughly. He therefore states that the first 75 cc. of the filtrate must be discarded for this reason, but that the portion following contains constant quantities of its various constituents. The same principle was applied to the filtration of the milk for tests for enzymes. Even after filtering 200 to 500 cc. of the milk solution, so that the filter should be very thoroughly saturated, the serum gave not the faintest trace of an enzyme test. When the thick deposit on the surface of the filter was scraped off and suspended in water, this mixture was found to give the enzyme test, showing that the enzyme had remained with the casein.

The question then arose as to the possible disposition of the enzyme bodies in the milk solution. Are they attached to the casein molecule, and thereby prevented from passing through the filter, or are they free in solution? If the former condition exists, and the enzymes are tied to casein molecules, it would be impossible for them to pass through the filter, since all the casein remains on it, as shown by tests for casein on the filtrate by precipitation with acid. The filtrate showed no cloudiness upon the addition of acid in small quantity, therefore, no more than traces of casein could be present in it. The application of the biuret test showed the presence of protein, but this was given by the lactalbumin and unknown proteins of the milk which had passed through the filter. If the enzymes are free in solution, and the solution containing them could be separated from the casein by precipitation with acid, the casein-free solution with its enzyme content might be forced through the filter. The acid chosen for this precipitation must be one which does not destroy the enzymes.

If we add a very small amount of any acid to milk the Schardinger reaction takes place much more slowly, even if the amount is not sufficient for the precipitation of the casein.[2] If enough is added so that all the casein is precipitated, then the Schardinger reaction does not take place at all. There are, then, in relation to the Schardinger enzyme, three possibilities:

(1) The enzyme is still in *solution*. If we filter through ordinary filter paper, we can observe the Schardinger reaction as soon as we have neutralized the excess of acid which hindered the reaction.

(2) With stronger acid we can *precipitate* the enzyme so that it is mixed with the casein. We can now still obtain a positive Schardinger test,

[1] *Loc. cit.*

[2] On the other hand a small amount of alkali increases the rapidity of the reaction.

if we neutralize the acid carefully without filtering. If we filter, even through filter paper, the serum is quite inactive.

(3) By the addition of still stronger acid we can alter the enzyme so much that it is not possible to get any Schardinger test, neither in the serum nor in the casein. We consider then the enzyme as being *destroyed.*

In these experiments care must be taken not to add too much alkali, because in alkaline solution methylene blue is reduced by milk sugar, and this reduction could be mistaken for the reaction of Schardinger. Therefore we made sure that this point was not reached. If we neutralized without filtration from the casein, we used just the amount of sodium carbonate, which corresponded to the amount of acid used. If we had filtered from casein we had of course to use less sodium carbonate, because casein is an acid itself. We added then sodium carbonate till the calcium salts of the milk began to be precipitated, that is, when the solution was about neutral towards litmus paper.

To avoid the possibility of a mistake, we made exactly the same operations with boiled milk and we never observed any decoloration of the methylene blue.

The amount of acid used for obtaining these 3 steps is not always absolutely constant. The first step was generally obtained by the addition of 0.4 cc. of saturated citric acid to 50 cc. of milk. (For the precipitation of the casein we need 0.3 to 0.4 cc. of citric acid. At the same time all the fat is precipitated with the casein, the fat emulsion being stable only in the colloidal casein solution.) The second step was observed after the addition of two or three times the stated amount of citric acid or after the addition of 0.4 cc. of 6 *N* sulfuric acid (29% H_2SO_4). The third step—destruction of the enzyme—was generally obtained by the addition of 2 cc. of citric acid or 0.8 cc. of sulfuric acid. Hydrochloric acid (6 *N*) seems to have considerably less action upon the enzyme than sulfuric acid.

In connection with the second step we might mention that it seems as if the enzyme could be separated by the acid into two compounds, the precipitate being inactive for itself if thoroughly washed with water, but becoming active again after mixing with the inactive serum. We have, however, not investigated this observation further.

After these preliminary experiments we precipitated the casein of one liter of milk according to step (1). Eight cc. of saturated citric acid were added. After filtration through paper and neutralization with sodium carbonate we obtained 100 cc. of a very active milk serum, the time for the Schardinger reaction being 1 min. 40 sec. to 1 min. 50 sec.

This milk serum passed through our Pasteur-Chamberland filter with ease, and the filter did not become clogged as before. But, unfortunately,

the filtrate gave no trace of enzyme test, and again it was indicated, even more clearly than before, that the enzyme body is too large to pass through the pores of the filter, or that it is of a nature which does not permit its filtration under the conditions used.

The next filter tried was an asbestos felt, prepared from shredded asbestos, and arranged upon a Büchner funnel. The layer of asbestos was about a quarter of an inch thick. The filtrate from this gave the enzyme test, but the time of reduction of the dye was not constant until 100 cc. of solution had been filtered. When well saturated, the asbestos gave a filtrate in which the enzyme content was constant. Thus the final aim of the paper was partially attained, since a filtered solution of the enzymes was obtained. Such a filter, however, does not hold back bacteria, so a sterilized milk could not be obtained from it.

The last possibility was that of a Berkfeld filter. Its pores are larger than those of the Pasteur filters, and the Berkfelds are almost germ-proof. The milk solution, freed from fat and casein by precipitation with acid, was filtered with ease through a Berkfeld under pressure. But the filtrate gave no enzyme test, and after the filtration of about 1500 cc., there was still no indication that the enzyme had passed through.

It was suggested that in alkaline solution the enzyme might pass through the filter, the alkali changing the condition of the enzyme body in a way to make it possible to filter it. Accordingly, some active whey was made alkaline with sodium carbonate, and filtered. The filtrate was neutralized carefully with citric acid, and the enzyme test applied to the neutral solution, but with negative results.

Summary and Conclusions.—The chief purpose for which the work was carried on was not accomplished. No method for filtering the enzymes was found from which a bacteria-free filtrate was obtained. The main conclusion must, therefore, be negative. There are a few observations to be noted, and conclusions may be drawn tentatively from them, though the limited number of facts concerning enzymes known certainly, upon which to base conclusions, makes them rather of the nature of suggestions.

The Schardinger enzyme is found to be water-soluble. That is, it remains in the liquid portion of the milk after the casein and the fat are removed by a small amount of acid. If we precipitate the casein by stronger acids, the enzyme is precipitated with the casein and by still more acid it is destroyed.

It is possible that the enzyme is united in some way with some other water-soluble component of the milk, and that it remains stable and active only as long as this unknown body remains stable. Such a body might be an albumin. Van Slyke's table of analysis of milk serum shows that only 37% of the albumin passes through the filter.

This, he says, may be due to the absorbing power of the casein molecules, which hold back the larger portion of it. If the enzymes are united to some albumin not passing through the filter, this union may account for their absence in the serum.

It is more probable, however, that they are separate bodies, having properties similar to those of the albumin, and of a closely allied nature. They remain active until the coagulation temperature of the lactalbumin is reached, 72–80°, and this is perhaps their own coagulation temperature.

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ORGANIC CHEMICAL REAGENTS. I. DIMETHYLGLYOXIME.

BY ROGER ADAMS AND OLIVER KAMM.

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The manufacture of less common organic chemicals at the University of Illinois[1] has reached the stage where much time is involved merely in preparing large amounts of certain substances, whose method of production has been thoroughly worked out. This part of the work is now occupying the full time of several chemists. The work at Illinois was taken up for two purposes: first, to supply chemicals which were entirely out of the market, and second, to give graduate students experience, partly in developing ordinary laboratory processes to large scale manufacture which might be directly transferred to plant production, and partly in devising new processes capable of development on a commercial scale. The second purpose is defeated as soon as the men must devote too large a portion of their energy to the routine manufacture of certain compounds. This difficulty could be avoided, however, if one or more chemical concerns would be willing to produce the most needed of these substances. Undoubtedly, the reason this has not yet been done is because of the scarcity of research chemists and their preoccupation with larger and more important problems.

In view of the above situation it has seemed advisable to publish full details of the preparation of several of the more useful organic chemical reagents which are being made at Illinois so that they may be available to any manufacturer, thus allowing him to produce these substances quickly and without the expensive research work which is ordinarily necessary. The materials will continue to be made at the University until such a time as the commercial concerns are able to put them on the market at a reasonable price. It is hoped that the publication of these preparations may also serve the second purpose of helping out the scientific institution which may prefer for one reason or another to make their own reagents.

[1] *J. Ind. & Eng. Chem.*, **9**, 685(1917); THIS JOURNAL, **40**, 869 (1918); *Science*, **57**, No. 1210, 225(1918).