EXPERIMENTAL
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**Milk Samples**

A number of samples of milk obtained from different cows belonging to Sahiwal, Red Sindhi and Tharparker breeds and from different buffaloes of Murrah breed from the cattle-yard of National Dairy Research Institute, Karnal, were examined. Several samples of cow and buffalo milk were also collected under personal supervision from different private dairies around Chandigarh. The breeds of these animals were not known. Some samples of goat milk were also collected from different localities of Karnal and Chandigarh.

**Analyses of milk samples**

The milk samples were stored in a refrigerator for 3 hours before doing stability tests and other analyses. Fat, protein and total solids contents were determined by Gerber, formol titration and gravimetric methods, respectively (5). Acidity was determined by titrating against sodium hydroxide (N/9) solution using phenolphthalein as the indicator. pH measurements were carried out using Beckmann pH meter with a glass electrode. The soluble and total calcium and magnesium contents were determined by the methods suggested by Davies and White (3). Estimations of citrate, phosphate and ash contents were carried out by the Indian Standard methods (5).

**Determination of heat stability**

Heat stability was determined by noting the time required by milk to clot on keeping it at 120°C. Two ml. milk was taken in a
sterilised pyrex test tube (10 x 1.5 cm.). The tube was sealed and held in a rack which was placed in a thermostatically controlled oil bath maintained at 120°C. Several such tubes, each containing a different sample, could be taken simultaneously. The tubes were given some motion occasionally in the bath to facilitate the detection of clot formation. The bath used was made of copper sheet, 0.2 cm. in thickness. The dimensions of the bath were: length 35 cm., breadth 25 cm. and depth 32 cm. Heating of the oil was done by using two immersion heaters of 750 and 500 watts. To begin with, both the heaters were switched on and the temperature was raised to 120°C. After that 750 watts heater was switched off and 500 watts heater was connected to a variable voltage transformer in order to reduce temperature fluctuations. The control heater was operated through an electronic relay by an adjustable mercury contact thermometer. The tube was withdrawn for a few seconds first after 30 minutes, then after the next 10 minutes and subsequently after every 2 minutes interval. It was inverted gently, reinverted and put back in the bath, if the milk was not coagulated. The time of coagulation was taken as a measure of the heat stability of the milk. When some reagents were added, the time of coagulation was reduced to a few minutes only. In such cases, the tube was withdrawn and replaced more frequently.

**Determination of rennet stability**

Recently automatic blood clot timer has been used by deMan and Batra (9,10) for measuring renneting time of whole, homogenised,
fresh and reconstituted skim milk. Macro film technique has been used by Sharma and Bhalerao (14,15). Some other techniques have also been described in the literature (6,8,11). However, in the present case, the visual method described by Berridge (1) and successfully used by Gall (4), Kirchmeier (7) and White and Davies (16), with some modifications, was preferred on account of its simplicity and a fair degree of reproducibility.

The rennet used was "1: 100,000" powder manufactured by E. Merck Darmstadt. Fifty ml. milk was taken in a sterilised pyrex glass tube (16 x 4 cm.) containing 10 mg. of rennet. The contents were shaken by gentle inversion and reinversion four times and then allowed to stand in an incubator maintained at 25°C. Another tube of smaller diameter was inserted to form a thin film between the sides of the two tubes. The coagulating time, taken as a measure of the rennet stability, was observed visually by noting the time required for the formation of a clot between the sides of the two tubes. The values were taken in duplicate and the mean was recorded. The time was taken to the nearest 0.1 minute. The values were reproducible within 2 per cent error.

**Determination of ethanol stability**

Two ml. milk was pipetted out in a sterilised test tube (10 x 1.5 cm.) and to this 2 ml. of ethanol solution in distilled water of a certain concentration was added. The contents were mixed by gentle inversion and reinversion of the test tube four times and
then poured into a sterilised petri-dish to see if clots were formed. If the test was negative, ethanol solution of increasing concentration was used and the observation repeated. The minimum concentration of ethanol required to give positive test was taken as a measure of ethanol stability.

The ethanol used was 'absolute alcohol' with density (20°C.) equal to 0.7968 g./ml. Several aqueous solutions were prepared using double distilled water.

Determination of flocculation values of electrolytes for milk

The flocculation values of a number of electrolytes (A.R. quality) of different valency types were determined by adding to 5 ml. portions of milk, contained in pyrex glass test tubes of 25 ml. capacity, an equal volume of salt solution of known concentration. The tube was closed by a clean and dry cork and the contents shaken gently for about a minute (moving up and down 15 times) and then allowed to stand in a thermostatically controlled water bath maintained at 25°C. (± 0.05°C.) for 15 minutes. The lowest concentration of the electrolyte at which turbidity appeared was noted. The pH value of the coagulum was determined with a glass electrode.

Preparation of casein sol

Casein sol used in flocculation studies described in chapter IV was prepared by taking one gram of casein separated from skim milk by Hammarsten method (p. 156) and dissolving it in 100 ml. of
0.02 N sodium hydroxide. The excess of alkali was removed by dialysis using cellophane membrane, till the pH of the sol was about 7.

**Cation exchanger**

The cation exchanger used was Na-, K- or Ba- charcoal (13). One hundred ml. of milk was mixed with different amounts, varying from 0.1 to 1.0 g., of the appropriate cation exchanger and the suspension shaken for 30 minutes to complete the exchange process.

**Determination of heat of flocculation of milk**

The calorimeter used in these investigations is shown in Fig. 1. It consists of a wide mouth Dewar flask, F, of 1.5 pint capacity, fitted with a rubber cork equipped with a highly sensitive Beckmann thermometer, T, and a pyrex glass tubing, G, through which passes another glass tubing, C, of a smaller bore. The latter carries a rubber stopper, S1, and rests over the upper end of the tubing, G. It is attached to a perforated stainless steel bulb-holder, H, at the bottom. The holder also serves as a stirrer. The rotation is caused by the loop of string passing over a groove round the stopper S1. The bulb, A, containing the flocculating solution, is enclosed in the holder with its sealed end downwards, as shown. A breaking rod, R, rests on the bulb, A, so that it can break the bulb on applying pressure from above. The rod is prevented from touching and breaking the bottom of the Dewar flask, after the bulb is broken, by the cork S2, which then rests on the upper end of the tube, C. This arrangement is shown more clearly in Fig. 1(a). The calorimeter was
FIG. 1 - THE CALORIMETER USED FOR DETERMINING HEAT OF FLOCCULATION.
It was ascertained in preliminary experiments that the rate of stirring of 30 - 40 revolutions per minute gave sufficient mixing of the reactants and a minimum heating effect due to friction.

The bulb containing the flocculating solution was broken by tapping the rod gently. Blank runs showed that the thermal effects resulting from this action was almost negligible. The heat capacity of the system was determined by passing a known quantity of current and noting the rise in temperature.

Procedure: The bulb, A, was thoroughly cleaned and dried and 10 ml. of the flocculating solution was put into it. Its open end was sealed by means of a low flame. One hundred ml. milk diluted with an equal volume of water was taken in the calorimeter. In the case of ethanol, 50 ml. of milk was taken in the calorimeter and the same volume of ethanol was taken in the bulb. The temperature of the thermostat was maintained at 30 + 0.05°C. throughout. Stirring was started and the calorimeter was allowed to attain the temperature equilibrium. The bulb was then broken inside the milk and the time-temperature readings were continued for about 20 minutes, although the maximum rise or fall of temperature was observed during the first 10 minutes.

The amounts of flocculents taken were such that these could cause almost instantaneous precipitation. The effect of adding higher concentrations of flocculents was studied in preliminary experiments but there was no significant change in the thermal values involved.
Control experiments, using the same volume of water instead of milk, were also carried out and necessary corrections applied.

**Casein samples**

Ten samples of casein were prepared, 5 from cow milk and 5 from buffalo milk; one sample of each variety was prepared by each one of the following methods:

- (a) By the addition of rennet
- (b) By the use of ultra-centrifuge
- (c) By Hammarsten method
- (d) By Zoller method
- (e) By Van Slyke and Bosworth method.

The details of these methods are given below:

(a) **Rennet method**: One litre skim milk was heated to 35-37°C. and enough of rennet (E. Merck 1:100,000) was added to cause coagulation in about half-an-hour. The curd was broken into small lumps by stirring mechanically after which the whey was separated by filtration and the mass was washed in a blender first with water and then with acetone to remove free water and finally with ether to remove acetone. It was finally dried over sulphuric acid in a vacuum desiccator.

(b) **Ultra-centrifuge method**: Sixty ml. of skim milk, taken in 6 tubes were whirled in an ultra-centrifuge @ 30,000 r.p.m. for a period of 30 minutes. The residue was washed first with water and then with acetone and ether, as in the rennet method, and finally dried over
sulphuric acid.

(c) **Hammarsten method**:- One litre skim milk diluted with 4 litre water was stirred mechanically in a glass trough while dilute acetic acid was added slowly through a dropping funnel until casein was precipitated out at its isoelectric pH value of 4.6. The precipitate was dissolved in ammonia and reprecipitated on the addition of dilute acetic acid. The mass was washed and dried as above.

(d) **Zoller method**:- The procedure was essentially the same as in the Hammarsten method except that an aqueous solution containing 5 per cent hydrochloric and 5 per cent citric acid was used as the precipitant. The casein was dissolved in excess of dilute HCl to bring down its pH value to 3.0 and was reprecipitated on raising the pH value to 4.6 on the addition of ammonia.

(e) **Van Slyke and Bosworth method**:- The procedure initially was the same as in the Hammarsten method except that instead of dissolving casein in hydrochloric acid it was dissolved in ammonia and sufficient ammonium oxalate was added to precipitate out calcium. Casein was reprecipitated from the solution on the addition of dilute hydrochloric acid to bring down the pH value to about 4.6.

**Determination of water sorption isotherms**

The water sorption isotherms were obtained by following the procedure standardised by Puri et al. (12). 0.2 g. portions of the sample under examination, taken in small watch glasses, were kept in vacuum desiccators containing sulphuric acid-water mixtures.
corresponding to different relative vapour pressures of water. The composition of these mixtures was taken from the International Critical Tables. The desiccators were allowed to stand in an incubator maintained at 30°C. (± 0.2°C).

The increase in weight of the samples was determined by withdrawing the watch glasses first on alternate days and then daily. At the time of weighing, the watch glass under examination was kept covered by another watch glass of known weight. Each weighing took about a minute or so, since the previous weight was known. Each sample, after weighing, was replaced in the desiccator from which it had been withdrawn. The adsorbents were considered to have attained equilibrium with the surrounding atmosphere when their weights became constant within 0.5 mg. The equilibrium was attained generally after 4 to 10 days. The amounts of water vapour adsorbed per 300 g. were computed and plotted against the corresponding relative vapour pressures in each case. The watch glasses themselves, when kept alone in the desiccators under similar experimental conditions, did not pick up moisture to any noticeable extent.

Determination of heats of dissolution in organic solvents

The isothermal phase-change calorimeter, based on an appreciable change in volume accompanying the melting of diphenyl ether at 26.9°C, first described by Dainton et al. (2), was suitably modified for the present experiments. The results obtained were reproducible within 0.02 cal. The apparatus is shown in Fig.2.
FIG. 2 - THE CALORIMETER USED FOR DETERMINING HEAT OF DISSOLUTION.
central vessel R is the mixing vessel which contains 50 ml. of the organic solvent and a small glass bulb B, holding a known weight, usually about 1 g. of the fat under examination. The vessel is closed with a hollow stopper S which is evacuated to minimize the loss of heat due to radiation. A glass stirrer passes through the centre of the stopper and carries small glass blades at its lower end. The same stirrer is used to break the bulb. The stopper is fitted with a mercury seal P to avoid the contact of the reactants with the atmosphere as well as to avoid the evaporation of organic liquid during the reaction.

The tube D, surrounding the mixing vessel R, contains the dilatometric fluid (diphenyl ether) which is supported above the level hh by a column of mercury, as shown in Fig. 2. At its base, the diphenyl ether jacket is connected to a glass spiral which is further connected to capillary C containing mercury. The capillary C is joined to a three way stop cock T, which in one direction connects it to the reservoir of mercury M and in the other direction to the horizontal calibrated capillary H of a uniform bore. The diphenyl ether jacket D is surrounded by vacuum jacket V.

The calorimeter, as described above, is placed in a water thermostat which is further enclosed in an air thermostat. The temperatures of water and air thermostats are maintained at 26.9°C, and the fluctuations of the temperature are reduced to less than ± 0.01°C.

The capillary H is tested for its uniformity by measuring the length of a known weight of mercury in different parts of the
capillary. The amount of mercury per cm. length of the uniform capillary is then calculated.

The position of the mercury in the capillary \( H \) is noted by means of a cathetometer reading up to 0.001 cm. The heating or cooling effect produced on account of the dissolution of the fat in the solvent is measured by the amount of diphenyl ether changing into liquid or solid state. This is indicated by the drift of the mercury column in the capillary \( H \). The apparatus is previously calibrated by studying the neutralisation reaction of hydrochloric acid against sodium hydroxide.

Procedure: The reaction vessel \( R \), was cleaned and dried by means of tissue papers and 50 ml. of the solvent maintained at a temperature of 26.9°C. was put into it. The bulb \( B \) containing a known weight of the fat was introduced in the vessel. After this, the mercury seal stopper was fitted on to the vessel in such a way that the sharp end of the stirrer rested gently on the glass bulb. After sometime the system attained a constant temperature. This was indicated by the mercury column remaining at rest in capillary \( H \). This usually took about 4 hours. The drift of the mercury column in the capillary \( H \) was read by means of a cathetometer. The bulb was then broken by gentle pressing of the stirrer. The stirrer was also set into rotation by hand so as to bring about an efficient mixing without producing any measurable heat change. The heat change developed on account of dissolution of the fat in the solvent was measured by the amount of diphenyl ether
changing from liquid into solid state (the equilibrium between the solid and liquid diphenyl ether was disturbed due to cooling effect developed as a result of the dissolution of fat). This was indicated by the drift of the mercury column in the capillary H. The drift was converted into calories; one cm. drift corresponding to 5.375 calories.

References


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