capillary tubes closed at the top and projecting above the liquid surface are placed in *B*.

In order to give an idea of the efficiency of this apparatus, the following may be stated:

Redistilled alcohol was put in *B* and a mixture of 10 cc. alcohol and 240 cc. water was boiled in the flask; when 10 cc. of distillate had been collected, the operation was stopped. The distillate proved to be 95.6-per cent alcohol and there was no alcohol in the residual liquid in the flask.

A mixture of 180 cc. aromatic sulfuric acid and 580 cc. water was placed in the flask and boiled. Ordinary alcohol was placed in *B*. At the end of each half-hour the thermometer readings and the volume of the distillate were noted, after which 5 cc. of the mixture were withdrawn from the flask, cooled, diluted and titrated, while the distillation was resumed. In Table X are given the results. The respective weights

TABLE X

Time Hrs.	Distil- late Cc.	Thermom- eter in vapor	Reading in boiling liquid	Weights of H_2SO_4 in 2 cc. of Liquid		Ratio of Free to Total H_2SO_4 Per cent
				Free	Combined	
0.0	0.0	78°	89.10°	0.0488	0.0343	79.35
0.5	43.50	78	91.40	0.0532	0.0328	79.51
1.0	80.00	78	93.50	0.0593	0.0314	82.70
1.5	109.20	78	95.90	0.0652	0.0304	84.10
2.0	129.60	78	97.90	0.0721	0.0255	86.93
2.5	141.80	78	99.30	0.0758	0.0231	88.34
3.0	148.70	78	99.80	0.0821	0.0181	91.44
3.5	152.20	78	100.10	0.0870	0.0152	92.56
4.0	154.30	74	100.30	0.0888	0.0147	92.89
4.5	155.80	69	100.50	0.0917	0.0118	94.31
5.0	156.30	60	100.70	0.0939	0.0093	95.49
5.5	156.90	60	100.85	0.0956	0.0088	95.77
6.0	157.20	50	101.00	0.0964	0.0073	96.46
6.5	157.50	48	101.15	0.0991	0.0050	97.64
7.0	157.70	48	101.30	0.1003	0.0034	98.34
7.5	157.80	48	101.45	0.1005	0.0029	98.58
8.0	158.40	48	101.70	0.1010	0.0019	99.05
8.5	158.80	48	101.85	0.1020	0.0015	99.52
9.0	159.00	48	101.90	0.1033	0.0003	99.86
9.5	159.00	48	102.05	0.1040	0.0000	100.00
10.0	159.00	41	102.10	0.1040	0.0000	100.00
10.5	159.00	40	102.10	0.1040	0.0000	100.00

of "free H_2SO_4 " and "combined H_2SO_4 " were obtained in this way: the 5 cc. of liquid withdrawn from the flask were made up to 25 cc. with water; of this dilute solution 10 cc. were titrated at once and 10 cc. were completely hydrolyzed in an open beaker on the water bath and then titrated. From the data thus secured the amount of free sulfuric acid and that of the ethyl-sulfuric acid present in each 2 cc. of the liquid in the flask are calculated.

It will be noted that throughout the distillation the temperature of the vapor in the side-tube *C* never rose above 78°; after the bulk of the alcohol had been removed, the reading of this thermometer slowly fell, since the quantity of vapor passing through *C* became less and less and the loss of heat through radiation from the thermometer bulb was not compensated for. At the same time the temperature of the boiling liquid slowly rose as alcohol was distilled off. The sum of the "free" and the "combined" acid also slowly increases, for the percentage of total acid in the boiling liquid rises as the alcohol is removed. Finally, we may note that under the conditions of the experiment over 9 hrs.' boiling was needed for the complete hydrolysis of the ethyl-sulfuric acid present.

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RENNIN—A NOTE AND A CORRECTION

By HOWARD T. GRABER

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Rennin is not official in the sense that it is recognized by either the United States Pharmacopoeia or the National Formulary, hence there is a wide variation in the article as found upon the market, as well as in the methods used for its standardization. It is universally recognized as a milk coagulant and all methods use milk as a medium for standardization.

In 1911 I published an article¹ entitled "Some Observations upon the Assay of Digestive Ferments." In this article I included the assay used by me in the standardization of rennin. There was an error in this script which has since been corrected. This error to which I want to call especial attention was in the amount of milk specified to be used for the test. It was just twice the volume intended, so that the results by this error were just half what they should be, or in other words the rennin seemed to be just half its real strength. I have tried other methods proposed, but I recommend the method as published by me both for its simplicity of operation and the reliability of the results obtained when one uses a standard of known value as control.

The enzyme rennin, like all other enzymes, is distinctly specific in its reaction and it shows its selective characteristics as to the nature of the proteid, temperature and reaction of the media as well as the presence or absence of organic and inorganic salts, especially those of calcium and the phosphates. Results often vary when samples of milk are used from different cows, as well as different lots from the same cow, and the best results are obtained when using a mixture from the milking of many cows. There also seems to be a seasonal variation in the ability of the milk to coagulate.

The following table gives the results of many of the tests:

DATE	ASSAY 1:30,000 in Min. Sec.		MONTHLY AVERAGE	DATE	ASSAY 1:30,000 in Min. Sec.		MONTHLY AVERAGE
4/ 1 to				11/ 5/15	10	30	
4/15/14			7.5	12/ 3/15	10	30	
8/ 7/14	10	30		22	11	30	
8	10	30	9	23	8		
10	7	30		24	10		10.5
15	7	30		27	13	30	
9/ 1/14	9		9	28	7	30	
1/24/15	16		13.75	29	11		
21	11	30		30	11		
2/16/15	8	30	9.25	31	11		
22	10			1/ 3/16	10	30	
3/ 8/15	10		11	4	11		
31	11			5	10		10.5
5/ 5/15	8	30		6	11	30	
13	11	10		7	11		
17	11		10.3	8	10		
27	10	45		2/14/16	11		11
29	10	45		3/ 6/16	10		
30	10	10		7	9	45	
6/ 1/15	9	5		8	7	30	
7	11			9	7	30	
12	8	30		10	5	30	
17	10	15	10.6	11	9	30	
22	12			12	9	30	7.5
22	12			14	8		
29	11	30		23	11	15	
7/ 2/15	8	45		24	7		
7	8		9.3	28	4		
9	8			29	6		
10	8	45		30	4		
21	12			31	7		
22	10						

Several years ago I selected a rennin to be used as a standard in the assay of this enzyme, and I have

¹ THIS JOURNAL, 3 (1911), 919.

followed it quite consistently ever since. In the spring of the year 1914 it was tested for a period of 14 days and was found to assay 1 : 30,000 in $7\frac{1}{2}$ min. and was accepted as such. In every assay made since then it has been run as a check upon the unknown sample.

There are 74 assays here enumerated extending over a period of about 2 years. A series of 14 assays in April, 1914, averaging $7\frac{1}{2}$ min.; a second series of 14 in December, 1915, and January, 1916, averaging $10\frac{1}{2}$ min., and a third series of 14 assays in March, 1916, averaging $7\frac{1}{2}$ min.

This is certainly a very interesting state of affairs and the natural question to be asked is, "What is the cause of this variation?" There are many causes: some are known and some we are still studying.

My experience seems to show that the rennin coagulates more quickly in the fall and spring than in the winter and summer, and this fact seems to prove my contention that there is a seasonable variation in the milk.

There may be several explanations of this phenomenon. The cattle may get more exercise and more organic and inorganic salts in the spring, summer and early fall, than in the winter, hence their milk supply may vary, especially in relation to its coagulability. If this be so, the question rises, why does not the summer supply show this same increment in coagulability? My answer is that it would if we could regulate the supply, but during the hot summer months the milk is so prone to temperature changes that this offsets the other advantages. Then, too, the period of lactation is an important consideration and milk will show great variations in composition during this time.

It is an established fact that the rennet coagulation proceeds in three¹ stages: (1) The calcium casein is changed into the para casein; (2) the rennet enzyme seems to act upon the insoluble calcium salts of the milk, converting them into a form sufficiently soluble to enable them to coagulate the calcium para casein; (3) the true coagulation.

It is the second change or conversion of insoluble calcium salts to soluble salts which seems to retard the progress of coagulation, and I have noticed that in those cases where the time of coagulation was prolonged, the viscosity of the milk gradually changes; it seems to thicken and acts as if it almost wants to coagulate, but something seems to hold it back—either calcium salts are lacking or the insoluble calcium salts are with difficulty made soluble and available. I have also noticed that if a milk, acting in this manner, is divided into two portions, to one of which a soluble calcium salt is added, the coagulation period is much advanced in this portion when compared with the portion to which no soluble calcium salt has been added. Hence my statement that the seasonal variation in milk is an important factor.

There are other factors which, without a doubt, influence this coagulation period. Various samples of milk will show a variation in the fat content and

although the large creameries standardize the per cent of fat in their milk, the smaller creameries do not, and the inclusion of a superabundance of fat globules in one sample of milk undoubtedly will have some bearing upon the ease of coagulation.

The effect of bacterial content is an important factor. The milk examined by the Detroit Board of Health ranges in bacterial count from 3,000 to 1,500,000 bacteria per cc., which variation must certainly exert some influence upon rennet action.

The range of reaction of the milk within the perceptible limits of litmus test is an important item for consideration. In some cases I have found that a milk which has started to sour and thus had developed acidity above normal, seems to accelerate the coagulation period of rennin; in other cases it has inhibited it.

The "hydrogen-ion" concentration, a fact overlooked in the past by most of us but receiving more or less consideration at present, may help us clear up some of these peculiarities in enzyme activity. I look forward to the time when we can eventually adopt a standard milk of normal hydrogen-ion concentration and adjust our enzyme solution to a hydrogen-ion concentration found to give its optimum activity, and thus have our method under absolute and scientific control.

Until such time as this arrives, the results here enumerated lead me to say, "Know your standard." Do not adopt it on a single test or even a single series of tests, but follow it through a series of months as I have mine, then assign a strength which seems a fair average.

The method I use for testing follows, with the correction before cited.

(1) Only fresh, whole, unpasteurized milk is to be used, preferably from a herd of many cows.

(2) *Milk*—Heat 2 qts. gradually to $43^{\circ}\text{C}.$; mix well and take 1 qt. for the unknown sample and the other for the standard. Do not overheat the milk and if by chance it has been heated high enough to produce a thin skin, discard and use a new lot.

(3) *Rennin Solution*—Dissolve 0.4843 g. rennin in 150 cc. distilled water, mix thoroughly by inversion gently, and take 10 cc. for a test (10 cc. upon 1 qt. of milk gives a proportion of 1 : 30,000). Do not shake the rennin solution violently or the agitation will injure the strength of rennin.

(4) Measure 10 cc. rennin solution into a wide-mouth precipitating jar of about $\frac{1}{2}$ gal. capacity; add 1 qt. of milk at $42^{\circ}\text{C}.$, mix thoroughly by pouring rennin solution and milk 3 times from one jar to another, taking time with stopwatch after the first pouring. The milk should now be just $40^{\circ}\text{C}.$ Keep the temperature at this point and test the milk for curd formation at half-minute intervals by tipping the precipitating jar forward gently; note the time required to separate the casein from the liquid whey; report the sample as 1 : 30,000 in this time.

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¹ Allen's "Commercial Organic Analysis," 8, 126.