

compounds may be deduced by considering the ease with which alcohols form addition-products with other metallic chlorides good examples of which for instance, are $\text{SnCl}_4 \cdot 2\text{C}_2\text{H}_5\text{OH}$ and $\text{SnCl}_4 \cdot 2\text{C}_2\text{H}_{11}\text{OH}$. When we treated stannic chloride with an excess of propyl alcohol we obtained no propyl chloride but a liquid boiling at 148° .

End-products of the $\text{PCl}_3 \rightleftharpoons \text{ROH}$ combinations have already been mentioned; their intermediate products, theoretically considered, may be many; these intermediate products may give directly by heating or under the influence of zinc chloride and heat, the required alkyl chloride. A study of these intermediate products will be made.

URBANA, ILL.

ON THE COMBINING POWER OF CASEIN WITH CERTAIN ACIDS.

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In several recent papers I have shown the amount of different alkalis which may be combined with a given weight of casein to form salts,¹ and have also pointed out the behavior of hydrochloric acid in combining with the products of casein hydrolysis brought about by prolonged digestion in presence of pepsin, with subsequent heating.² These results suggested another line of enquiry, *viz*:--the combining behavior of the acid, without the aid of pepsin, at the ordinary temperature as well as at higher temperatures. The amount of acid combining at the ordinary temperatures may in many cases be found by simple titration, while for higher temperatures other means may be employed.

At the ordinary temperatures many dilute acids combine with casein as with other proteins to form salts, and the amount of acid so held is definite and constant. By warming, however, or by prolonged contact, the acids lead to the formation of hydrolysis products, some of which as amino acids, are strong enough to hold considerable quantities of the acids used as the combining and hydrolyzing agents. It was shown in the last papers quoted that the amount of hydrochloric acid held in this way is about what might be expected from the character of the amino acid groups probably developed. But in the present inquiry we are concerned with the activity of the acids when not assisted by the digesting power of the pepsin, and the first experiments were made with $\text{N}/10$ hydrochloric acid at the ordinary temperature, followed by some in which the combination was effected by heat.

Hydrochloric Acid.—A series of tests was made, using always 5 grams of pure casein prepared by the Hammarsten process and varying amounts of the weak acid, with water enough to make up always 100 cubic centimeters. The mixtures of weak acid and casein were allowed to stand

¹ This Journal, 27, 363; 28, 372.

² Ibid. 29, 223; 29, 295.

about an hour with frequent shaking, and were then filtered, which, up to a certain strength of the acid, was readily possible. With stronger acid mixtures, however, a jelly-like mass was formed which could not be filtered rapidly or perfectly enough for the purpose.

When a filtrate could be secured, it was used for titrations with dimethylaminoazo benzene, phenol-phthalein and *p*-nitro-phenol, in most cases. Conductivity tests were frequently made on a part of the filtrate. In the mixtures which became too thick for filtration the whole was used for the titrations directly. The results obtained are shown in the following table, where the result of titrations with 25 cc. are reduced to 100 cc. for comparison :

TABLE 1.—5 GM. CASEIN + HCl.

CC. N/10 HCl	CC. Water	CC. N/10 NaOH. Orange	CC. N/10 NaOH. Phenol-phth	CC. N/10 NaOH. <i>p</i> -Nitro-phenol	Conductivity K_{20}
5	95	0.0	2.8	2.4	0.000275
10	90	0.0	4.9	4.0	0.000445
15	85	0.0	12.0	...	0.000739
20	80	0.0	16.0	...	0.000946
..
25	75	0.0	25.0	25.0	Jelly, titrated
30	70	0.0	30.0	30.0	whole mixture
35	65	trace	35.0	35.0	"
40	60	4+	40.0	40.0	"

It is evident from the table that the acid forms a soluble combination with the casein, and that for 5 gm. about 35 cc. of N/10 acid can be so held. With only 20 cc. of acid present, the mixture can be filtered. In the filtrate no "free" acid can be detected with the dimethylaminoazo benzene (which, in what follows, will be referred to as "orange"), but with phenolphthalein a large amount of alkali is required. This corresponds to combined acid and to the casein held with it also. The conductivity tests made on the filtrate show likewise a large amount of combined acid in solution. In titrating the jelly-like mixture, where more than 20 cc. of acid was used, the whole of this acid in combination may be recognized by aid of phenolphthalein, and with a fair degree of accuracy, too, if the titration is made quickly. The union of the combined acid with the added alkali is immediate and the reaction apparently becomes complete before combination of the liberated casein with further alkali begins. It follows therefore that a permanent color with phenolphthalein cannot be secured until the whole of the casein has gone into solution to form what I have described, in an earlier paper referred to as a "neutral" salt. This last reaction is somewhat slow, and it is possible to recognize rather easily when it begins. The reaction with the para nitro phenol is somewhat sharper here than is that with phenolphthalein.

In this reaction without the aid of heat 1 gm. of casein combines with about 7 cc. of N/10 HCl, or with about 25.5 mg. of the real acid. This combination is effected with the slightest possible conversion of the casein

into smaller groups, and is doubtless due to the presence of the free amino groups of the casein itself. It is evident that a reaction at a higher temperature, accompanied by hydrolysis, would result in the combination of a much larger amount of acid because of the presence of *liberated* amino groups. This is shown in the experiments with the acid and pepsin already referred to, where about ten times as much acid as here found was combined. The combining power without the aid of pepsin was determined in a new set of experiments in which casein and dilute acid were mixed, evaporated on the water-bath, dried at 102°-105° and weighed. The residue was then ashed after addition of sodium carbonate and the chlorine found in the ash. In this series of experiments the constant weight of 1.87 gm. of anhydrous casein (2 gm. of air-dry casein) with increasing weights of acid used in N/10 strength, were taken in each case.

TABLE 2.—1.87 GM. CASEIN + HCl, HEATED.

Mg of HCl added	Increase in weight	HCl in dry residue	Water added, calc.
109.5	129.5	105.9	25.6
146.0	211.0	136.9	74.1
164.2	204.0	133.2	70.8
235.5	231.5	160.6	70.9
273.8	245.0	148.2	96.8
292.0	260.0	164.3	95.7
310.0	265.0	157.0	108.0
328.5	296.5	182.5	114.0
346.8	252.5	160.6	91.9
365.0	267.0	158.8	108.2

These results appear somewhat irregular, but this is doubtless due to the method of evaporation followed. They show, in general, that at the temperature employed casein will combine with about one-tenth its weight of hydrochloric acid. This seems to be the maximum value for the conditions of the test. It is perhaps better to say that casein and its hydrolysis products have this value. The water added is calculated from the difference in weight between the numbers of the second and third columns of the table and in the mean amounts to about two-thirds of the acid addition in the last six tests. In these tests the ratio of the acid to water added is 161.9:102.4, which is nearly in the proportion of 4 molecules of acid to 5 molecules of water. This water addition points to a comparatively advanced degree of hydrolysis.

Hydrobromic Acid.—The experience with hydrochloric acid suggested some similar tests with the analogous hydrobromic acid. A solution of this acid of N/10 strength was made and employed in the tests with a constant weight of casein, using in the titrations the "orange" indicator.

TABLE 3.—5 GM. CASEIN + HBr

CC. N/10 HBr	CC. H ₂ O	CC. N/10 NaOH. Orange
20	80	0.0
25	75	0.0
30	70	0.0
35	65	trace
40	60	6.0—

It appears that slightly less than 35 cc. of the N/10 acid combines with the casein. On adding 40 cc. of acid and titrating back the reaction is not very sharp, but the addition of more than 5 cc. of alkali can be noted. This is in good agreement with the findings with the hydrochloric acid. To this last mixture phenolphthalein was added and then more alkali. The effect of the latter is to combine with the acid and then gradually dissolve the casein, which, by slight warming, may be accomplished in a short time. In this case, however, no heat was applied and an hour or more was required for complete solution. The N/10 sodium hydroxide was added in small portions as fast as the color disappeared through neutralization of the acid present and union with the acid group of the casein. The final permanent pink tinge from this indicator and the alkali was secured after the addition of nearly 86 cc. of the latter. As 40 cc. of N/10 alkali was used up in combining with the 40 cc. of N/10 hydrobromic acid added at the start, the remaining 46 cc. must have been used to combine with the casein. This is the amount practically required for 5 gm. of pure casein, as found by numerous previous experiments, and the test shows that in the hydrobromic acid combination there has been no essential change by hydrolysis to alter the combining proportions of the casein.

The combination of the casein with this acid forms a gelatinous, partly soluble mass which cannot be well filtered. The above tests were made therefore on the whole mixtures.

It was next attempted to find the amount of hydrobromic acid which combines with casein by aid of heat, applied during the evaporation of a solution in contact with the latter. In each case 1.87 gm. of pure casein was mixed with the acid in a small weighed porcelain dish and heated on the water-bath to complete dryness. The dishes were transferred to an air oven and heated half an hour to 102°-105° and weighed. The residues were ashed after addition of sodium carbonate and nitrate, and the bromine determined by the Volhard method. The following results were secured:

TABLE 4.—1.87 GM. CASEIN + HBr, HEATED.

Mg. of HBr added	Increase in weight	HBr in dry residue	Water added calc.
283.5	318.5	274.4	43.8
324.0	353.0	307.8	45.2
364.5	409.5	355.5	54.0
405.0	467.0	377.7	89.3
445.5	522.0	405.0	117.0
486.0	551.5	469.8	81.7
526.5	568.5	470.0	98.5
567.0	611.5	502.2	109.3
607.5	649.0	542.7	106.3
648.0	686.8	558.9	127.9
688.5	751.5	631.8	119.7
729.0	771.0	632.0	139.0
769.5	798.5	664.2	134.3
801.0	809.5	639.9	169.6

Like the figures for the combination with hydrochloric acid the above are somewhat irregular. But they indicate a progressive addition of hydrobromic acid, amounting finally to about 35 per cent. of the weight of the pure casein. This very large increase in weight can follow, probably, only through partial hydrolysis and union with the resulting amino groups. In general, as indicated by the water addition shown in the last column, the extent of hydrolysis is much greater than with the corresponding reaction in which hydrochloric acid was used, although not as great as found for the latter combined with pepsin. No reason suggests itself for the greater activity of the hydrobromic acid.

Hydriodic Acid.—The experiments with this acid were carried out exactly as with the other halogen acids, but it was found that in the direct titration process the point at which no more acid combined with casein could not be as clearly recognized. When titrated by aid of methyl-orange and dimethylaminoazo benzene it was found that 1 gm. of pure dry casein combined with slightly less than 7 cc. N/10 HCl or HBr. The hydriodic acid is not as stable in aqueous solution as are the other acids and the traces of dissociation products from the casein seem to interfere somewhat with the delicacy of the end reaction in the titration. A number of experiments made, however, show that approximately 35 cc. of N/10 acid combine with 5 gm. of pure casein, which is the combining proportion of the other acids.

While the reaction at the ordinary temperature is not as sharp as in the other cases, that which follows by aid of heat appears to be about the same, as shown by the table which follows. In these experiments, as before, 1.87 gm. of dry casein was evaporated with increasing weights of N/10 hydriodic acid and the perfectly dry residue weighed. The combined iodine was then found in the ash of the fusion with sodium carbonate.

TABLE 5.—1.87 GM. CASEIN - HI, HEATED.

Mg. of HI added	Increase in weight	HI in dry residue	Water added, calc.
512	527.0	502.0	25.0
576	602.0	512.0	90.0
640	664.5	531.2	133.2
704	711.5	601.6	109.9
768	767.5
832	857.0
896	933.5	768.0	165.5
960	1013.5	870.4	143.1
1024	1086.5	921.6	164.9
1088	1135.5	857.6	277.9
1152	1223.0	966.4	256.6
1216	1269.5	1070.0	199.0
1280	1325.0	1075.0	250.0

While the numbers expressing the increase in weight are pretty regu-

lar, those measuring the iodine added are not. The reason for this is not hard to give, however. The iodine was determined from a fusion process in which there was some loss, notwithstanding the presence of the sodium carbonate. The products before fusion are quite dark and hard, but may be ground down to a fine brownish powder. During the evaporation the mixtures present a gelatinous appearance, but not as marked as in the case of the hydrochloride. Some degree of solubility follows, and even the dried mass is partly soluble in water.

The product resembles very closely several medicinal preparations which have been placed on the market in recent years, but under names which suggest an origin other than from casein. This substance is undoubtedly the starting material in the manufacture of many of these compounds which are advertised to contain 20 to 25 per cent. of iodine. In the last of the combinations of the above table we have a content of over 33 per cent. of iodine as iodide. This probably does not represent the maximum combining power, but merely that corresponding to a certain degree of hydrolysis. It is interesting to note that the additions of hydrobromic and hydriodic acids are very nearly in molecular proportions.

Sulphuric Acid.—As distinguished from the halogen acid combinations those between sulphuric acid and casein are less soluble. While the halogen acids form compounds which behave like jellies the sulphuric acid compounds remain finely granular, and if stirred up with water soon deposit the apparently unchanged casein in a form easily separated by filtration. A series of mixtures was made as before and, after standing, the filtered supernatant liquid was used for titrations, 25 cc. being taken for each test. The results are reduced to 100 cc. for comparison.

TABLE 6.—5 GM. CASEIN + H₂SO₄

CC. N/10 H ₂ SO ₄	CC. H ₂ O	CC. N/10 NaOH. Orange	CC. N/10 NaOH. Phenolphthalein	CC N/10 NaOH <i>p</i> -Nitrophenol	Cond. κ ₂₀
5	95	0.0	1.2	0.8	0.000154
10	90	0.0	1.6	1.2	0.000184
20	80	0.0	2.6	2.0	0.000304
30	70	0.0	4.0	3.8	0.000666
40	60	4.4+	7.2	6.8	0.001737
50	50	13.0+	15.2	13.2	0.003759

The titrations with the "orange" show that a little over 7 cc. of N/10 H₂SO₄ must be used to neutralize 1 gm. of casein by this method, which result is essentially the same as found for the halogen acids. The values obtained with phenolphthalein and *p*-nitrophenol have the same meaning as with the halogen acids. They represent the acid combined with the casein which has gone into solution and also the casein itself, but as the reaction for the latter is not absolutely sharp, the values are somewhat irregular.

The values given under the head of conductivity show also that the sulphate is not perfectly insoluble, but evidently much less soluble than the halogen compounds. This conductivity is not wholly due to the salt formed, however. On mixing 100 cc. of the distilled water used in all the experiments with 5 gm. of casein, and filtering through the Swedish filter papers employed in all the tests, a filtrate was secured which showed $\kappa_{20} = 0.0000484$. The above result would have to be diminished by approximately this amount to find the true conductivity of the dissolved casein salt. The remainder is quite appreciable for the larger acid volumes used, because here much remains free.

No attempt was made to find the combining value of sulphuric acid at the higher temperature, because of the charring which follows on concentration.

Acetic Acid.—This acid forms a feeble combination with casein and unites approximately in the same proportion found for the other acids, as just described. It is well known that the destructive action of this acid on casein is far less than with the mineral acids, and advantage is taken of this in the preparation of pure casein by precipitation from milk.

A number of titrations were made as in the other cases using N/10 acetic acid with water, and 5 gm. of casein in each test. The mixtures were frequently shaken and filtered after an hour or more. As before the titrations were made on 25 cc., but the results are calculated to 100 cc.

TABLE 7.—5 GM. CASEIN -- $\text{HC}_2\text{H}_3\text{O}_2$

CC. N/10 $\text{HC}_2\text{H}_3\text{O}_2$	CC. H_2O	CC. N/10 NaOH Orange	CC. N/10 NaOH Phenolphthalein	CC. N/10 NaOH <i>p</i> -nitro-phenol	Cond. κ_{20}
5	95	0.0	5.2	3.6	0.0000956
15	85	0.0	14.8	13.9	0.0001523
25	75	0.0	24.8	23.2	0.0001916
35	65	?	34.0	28.0	0.0002238
45	55	end not sharp	44.0	43.0	0.0002586

The acetic acid does not form a jelly with the casein, but behaves as does sulphuric acid. From the titration and conductivity values it is evident that a relatively large amount of the solid goes into solution. The titration values with phenolphthalein measure the alkali required to combine with the acid added, and also with the liberated casein, after being set free from the acid in the filtrate. About one-half of the alkali run into the first four filtrates, is used for each combination, since in titration the first drop of alkali added produces a turbidity. This turbidity increases and then disappears when enough alkali has been added to form first an acetate, and then the usual neutral salt with the casein, as shown by the permanent phenolphthalein color.

An attempt was made to find the increase in weight which follows on drying down a given weight of casein with the solution of acetic acid. The tests were carried out as in the other cases, but it was not possible

o determine the combined acid in the dry residue, because of lack of delicacy in the method. The actual weight increase is given in the following table, the weighings being made after drying at 102-105°. The appearance of the dry residues suggested the formation of hydrolysis products similar to those secured in the other cases.

TABLE 8.—1.87 GM. CASEIN + $\text{HC}_2\text{H}_3\text{O}_2$, HEATED.

Mg. of $\text{HC}_2\text{H}_3\text{O}_2$ added.	Increase in Weight.
30	18.0
60	27.5
90	27.0
120	46.5
150	53.0
180	78.0
210	88.0
240	94.0
270	141.0
300	118.0

Oxalic and Tartaric Acids.—These acids combine with casein and form salts of rather marked solubility, but as the determination of the free acids in presence of the casein cannot be sharply made, the actual combining power was not found.

In the evaporation of mixtures of casein with these acids there is evidently considerable hydrolysis, and apparently combination with the acids. But on account of the non-volatility of the excess of acid used, it is not possible to fix very exact limits for the combination here.

Phosphoric Acid.—This acid in weak solution has a very marked action on casein. The combination forms a jelly almost immediately, and this dissolves in water to a considerable extent. The rapidity of jelly formation is even greater than with the halogen acids. On evaporation of such mixtures and drying at 105°, the increases in weight are in excess of the solid added in the form of phosphoric acid, pointing to a considerable fixation of water. Because of the lack of delicacy in the indicators, no satisfactory results were obtained in titrating the excess of acid left after mixing casein with the latter in the cold.

Boric Acid.—There appears to be no combination of this acid with casein. On mixing the latter with solutions of the acid, a separation soon follows, and the total amount of free acid seems to be found in the supernatant liquid. It was found, also, that after evaporating mixtures of casein with boric acid solutions the residue left contained, with the casein, free boric acid only, readily extracted with water and alcohol.

Behavior of p-Nitro-Phenol.—It will be observed that *p*-nitro-phenol was employed in a number of the above titrations, and that the results obtained through this indicator are, in most cases, similar to, but not quite identical with those found with phenolphthalein. The color

change with the *p*-nitro-phenol is as rapid and as sharp as with the other in the mixtures titrated. It is evident, therefore, that in using this indicator, we are able to measure not only the free mineral acids, but the acid combined with casein also.

In the titration of the casein itself, considered as an acid, the range of the phenolphthalein is wider than that of the *p*-nitro-phenol, as illustrated by the results in the acetic acid experiments. This difference is possibly due to the fact that we have to deal with some decomposition products of casein in small amount, and the acid strength of some of these may not be sufficient to show with the *p*-nitro-phenol. This indicator may have practical value in differentiating between just such mixtures, but as yet its full importance cannot be assigned.

Conclusions.

These experiments show that the basic behavior of casein toward acid is as marked and definite as the acid behavior toward alkalies. At the ordinary temperature one gram of dry casein combines with nearly 7 cc. of *N*/10 hydrochloric, hydrobromic, hydriodic, sulphuric and acetic acids, and also with tartaric, phosphoric, oxalic and other acids for which numerical values could not be found. The base fixing-power is somewhat greater, as one gram combines with 9 cc. of *N*/10 sodium, potassium, lithium or ammonium hydroxide or carbonate.

With application of heat, as in the evaporation of weak acid solutions with casein, the combining power becomes much greater. For hydrochloric acid about four times as much acid appears to combine at the higher temperature as was found in the cold titration. For hydrobromic and hydriodic acids the combining rates at high temperature are relatively greater. This may be due to the simple effect of the heat in favoring the union with additional amino groups of the casein, but it is undoubtedly due, in part at least, to the formation of and combination with the products of hydrolysis.

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STUDIES OF THE NITROGEN CONTENT OF WHEAT AND ITS DISTRIBUTION TO DIFFERENT PARTS OF AN INDIVIDUAL PLANT.

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The following article is a brief report of the results of a study of the distribution of nitrogen in the several parts of the different heads of wheat which grow from a single seed, and hence form a single individual wheat plant. The object of the study was two-fold; first, what might be termed an anatomical study to show how the plant distributes its nitro-