Anal. Calcd. for $C_7H_8O_5N_2$: C, 42.0; H, 4.0; N, 14.0. Found: C, 42.18, 42.4; H, 4.51; N, 13.27, 13.18.

2,6-Dioxy-5-hydroxypyrimidine (Isobarbituric Acid), XVIII.—One g. of 2-oxy-4-carboxylic acid 5-ethoxy-6-oxypyrimidine was sealed in a tube with 10 cc. of concentrated hydrochloric acid and heated for two hours at 110°. Under these conditions no reaction took place. It was therefore heated again for ten hours at 160–165°. The reaction product was found to be insoluble in water and also difficultly soluble in alcohol. On recrystallization from alcohol it separated in the form of microscopic crystals arranged in clusters. These decomposed without melting at 350–355°. The compound was identified as isobarbituric acid. In other words, the carboxyl group is removed by intensive hydrolysis, the pyrimidine behaving in an entirely different manner than uracil-4-carboxylic acid,[2] which resists hydrolysis when heated with 20% sulfuric acid at 200°.

Anal. Calcd. for $C_5H_4O_5N_2$: N, 16.27; for $C_4H_4O_3N_2$: N, 21.87. Found: N, 21.71.

Summary

1. Isouracil-carboxylic acid can exist theoretically in several isomeric modifications and is isomeric with *orotic acid*, a substance stated to have been isolated from milk by Biscaro and Belloni.

2. Experimental evidence has been obtained indicating that isouracil-carboxylic acid may be identical with orotic acid.

3. Two different methods of synthesis have been applied for preparing isouracil-carboxylic acid. Only one thus far gives promise of leading successfully to this pyrimidine.

4. Several new derivatives of isouracil have been prepared.

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THE SEPARATION OF THE SOLUBLE PROTEINS OF RABBIT MUSCLE

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RECEIVED OCTOBER 27, 1928 PUBLISHED MARCH 6, 1929

The earlier work on the separation of muscle proteins has been adequately reviewed,[1] so we will omit a detailed report of those investigations. Howe[2] has recently described a very ingenious procedure which deserves further study, in order to define more precisely the limits of its usefulness. There are several other recent papers[3] that have some bearing on the problem of extracting and separating muscle proteins, though they do not con-

[1] G. Mann, "Chemistry of the Proteids," Macmillan and Co., London, **1906**.

[2] Howe, *J. Biol. Chem.*, **61**, 493 (1924).

[3] Lloyd, *Proc. Roy. Soc. London*, **89B**, 277 (1916); Collip, *J. Biol. Chem.*, **50**, xlv (1922); Granstrom, *Biochem. Z.*, **134**, 589 (1922); Weber, *ibid.*, **158**, 443 (1925); **184**, 407 (1927); Salter, *Proc. Soc. Exptl. Biol. Med.*, **24**, 116 (1926); Wohlisch and Schriever, *Z. Biol.*, **83**, 265 (1925).

tribute directly to our knowledge of the quantities that may be obtained. Lack of space, however, prevents more than mere mention.

Experimental

For our preliminary observations it seemed necessary to have samples that were as uniform as could be obtained. The material should be readily available, the tissue should be fresh and reasonably free from blood, so rabbits were chosen as experimental animals. They were killed by striking them a sharp blow on the back of the neck and the posterior portion of the animal was perfused with a Ringer-Locke solution until the perfusing liquid was free from color. At this point the rabbit was quickly skinned, the hind quarters removed and the tissue needed separated from the bone. We have assumed that complete removal of the soluble muscle proteins cannot be accomplished unless the tissue is finely subdivided, and have used two methods of grinding, either of which we regard as at least fairly satisfactory. When we first began, the samples were frozen with carbon dioxide and ground in a mortar. A somewhat higher percentage of extraction was obtained when samples were ground in this way but the procedure was tedious, and as the degree of extraction was not of primary importance at the time, this method of grinding was discontinued. Samples were next ground in a Nixtamal mill, which gives a very finely divided sample.

Methods of Extraction.—The samples, either 100 or 125 g. in size, were equally divided between four bottles of 200-cc. capacity and 100 cc. of the extracting solution was added to each bottle. These were gently shaken and allowed to stand for about an hour. The bottles were then centrifuged until the solid material had been thrown down making it possible to pour off the supernatant liquid without loss of tissue and the procedure was repeated until we were ready to discontinue extraction. When the extraction was complete the extract was made to volume, 2.0 liters for a 100-g. sample, 2.5 liters for a 125-g. sample, and filtered through a dry pad of cellucotton on a Büchner funnel. It has been reported frequently that muscle albumin rapidly changes its properties if allowed to stand at ordinary temperatures. For that reason the original samples, and all extracts, were constantly kept packed in a mixture of ice and salt. Nitrogen was determined in the original sample of muscle and in aliquots of the extract.

In studies such as we are reporting the choice of a solvent is of considerable importance, so we tried a large variety of solutions. Some of these were promising, but for various reasons we decided to use only 10% sodium chloride. It uniformly yielded a percentage extraction as high or higher than the other solutions, with the exception of ammonium chloride. In addition we had in mind the possibility of studying the separated proteins by the use of titration curves, and we wished to use a salt that has no buffer action. The hydrogen-ion concentration of the extracting solution was adjusted to a P_H value of about 6, varying between 5.9 and 6.1. The electrometric method was used almost exclusively in making hydrogen-ion determinations.

Separation of Protein.—In our studies of the forms of nitrogen present in muscle extracts, we have considered only three fractions, designated by us as albumin, globulin and non-protein nitrogen. In our preliminary observations various methods of precipitating the globulin were tried. Several of these offer promise but we are only reporting three in any detail: salting-out, dialysis and irradiation with a quartz mercury arc.

As to the salting-out process, several different salts seemed suitable for the purpose and our final choice of sodium chloride was based chiefly on convenience. An additional reason was that the same salt had been used as the solvent.

During our efforts to develop a satisfactory technique for precipitation by dialysis, we attempted the method of electro-dialysis. At first this procedure was regarded as very promising, but it was soon discarded, due to the difficulty of controlling the hydrogen-ion concentration. We finally decided to try dialysis by the usual method, taking such precautions as we might to prevent changes in the solubility of the protein. The dialyzing cell was immersed in ice water and toluene and thymol were added to prevent bacterial action. We believe these precautions are largely effective.

So far as we are aware, exposure to ultraviolet rays has never been used as a method of precipitating proteins quantitatively, though the effect of rays of short wave length on proteins has been reported by a number of investigators. Most of the publications have been reviewed by Clark[4] and by Stedman and Mendel[5] and we are omitting any discussion of individual reports. These contain many discrepancies but there is almost complete agreement on one point: protein solutions are rendered more unstable by irradiation. Some proteins are much more affected than others; for example, albumins are less readily coagulated by irradiation than are globulins. It occurred to us that if our muscle extracts contain two proteins, one might be coagulated more quickly than the other and so separated more or less quantitatively. We made no effort to determine whether or not the effects observed were due entirely to ultraviolet rays. The work of Young[6] suggests that visible rays also may bring about marked changes in the properties of protein solutions.

Precipitation of Muscle Globulin by Saturation with Sodium Chloride.—Since albumins in a concentrated salt solution are precipitated by the addition of acid, it seemed advisable first to determine within what range of hydrogen-ion concentration the globulin could be precipitated without contamination with albumin. Apparently the same amount of protein is precipitated at a P_H of 6, 7 or 8. Distinctly larger amounts are precipitated at a P_H of 5. From the standpoint of convenience in manipulation, a P_H of 6 is to be preferred, as the precipitate is always granular in character and easily washed on the filter. At a P_H of 7 the precipitate is slightly gelatinous at times, and at a P_H of 8 it is distinctly slimy and the washing process is very slow. The procedure of salt separation is well known, so we are omitting details of the technique we employed. Typical results are given in Table I.

[4] Clark, *Am. J. Physiol.*, **73**, 649 (1926).

[5] Stedman and Mendel, *ibid.*, **77**, 199 (1926).

[6] Young, *Proc. Roy. Soc. London*, **93B**, 235 (1922).

TABLE I

PROTEIN IN EXTRACT PRECIPITATED BY SATURATION WITH SODIUM CHLORIDE

Date	PH 4	5	6	7	8
12/29/26			61.3	56.3	
1/28/27	80.7	85.9	62.3	59.7	59.7
2/23/27	82.8	79.4	60.3	61.8	64.0
3/15/27	75.3	70.8	60.6	51.2	52.7
4/ 4/27	84.3	77.5	61.8	57.8	62.1
4/18/27		78.1	59.4	57.7	
5/10/27		73.8	57.7		
6/ 1/27		80.7	60.8		
9/28/27		80.1	61.4	57.3	
10/17/27		65.6	59.3	50.1	
12/10/27		69.2	56.7	54.5	
12/30/27		78.1	60.9	61.1	
1/30/28		69.3	53.3	53.3	

Separation of Globulin by Dialysis.—Parchment chambers were used for dialyzing cells and in practically every case four aliquots were taken. They were dialyzed against tap water for twenty-four hours, against distilled water for twenty-four hours and then for another twenty-four hours against distilled water of a definite hydrogen-ion concentration. In terms of *P*H units these were 4, 5, 6 and 7. After dialysis was complete, the precipitate was used for a nitrogen determination; also, an aliquot of the filtrate was saturated with sodium chloride and the nitrogen content of the precipitate was determined. The sum of these determinations was taken as representing the total globulin. We have explained the possible presence of globulin in the filtrate by assuming that it is held in solution by the albumin or by the non-protein constituents. After dialysis the solution does not give a test for chlorides.

The amount of protein precipitated seemed to be unrelated to the hydrogen-ion concentration of the dialysate, for the amount of protein precipitated in the four cells was approximately the same. There was considerable variation in individual determinations, however, as is shown in Table II.

TABLE II

PROTEIN IN EXTRACT PRECIPITATED BY DIALYSIS

PH	Date 2/23/27	3/15/27	4/4/27	4/18/27	5/10/27	10/17/27	12/10/27	1/30/28
4	50.2	54.0	58.9	58.8	64.6	52.2	60.4	63.2
5	51.2	53.3	53.4	60.9	58.7	54.4	64.8	64.7
6	..	50.6	65.8	59.9	55.9	57.5	67.7	66.3
7	48.0	53.1	57.6	55.8	57.6	57.3	..	66.0

Precipitation by Exposure to Ultraviolet Rays.—A preliminary trial made it quite evident that protein is precipitated from solution by irradiation with a quartz mercury arc, so we attempted to determine whether the

method could be used for the quantitative estimation of muscle globulin. Our experience was that the hydrogen-ion concentration of the liquid had to be controlled quite rigidly if precipitation by irradiation was to be satisfactory. At a P_H of 4 or 5 the quantity of protein precipitated was definitely too high. Best results were obtained at a P_H of approximately 6, as precipitation was quite rapid and the precipitate was flocculent and easily washed. At a P_H of 7 or 8 a longer time was required for precipitation and the precipitates were too gelatinous to be filtered and washed satisfactorily. In order to obtain more definite information as to the time required for precipitation, extracts at a P_H of 5.8 were exposed for variable periods of time, one, two, four, six and eight hours. It developed that irradiation for four hours or less is too short a period, and that the quantity of protein precipitated during six or eight hours is about the same.

Determination of Albumin.—The filtrates obtained by the three different procedures for globulin precipitation varied widely in their salt content but all were treated alike for the precipitation of the albumin. They were heated to boiling and sufficient trichloro-acetic acid was added to make the final concentration 2.5%. The nitrogen content of the precipitate was then determined.

Determination of Non-Protein Nitrogen.—The filtrates from the albumin precipitates were taken for nitrogen determinations. This fraction was designated as non-protein nitrogen.

Comparison of Procedures.—Thus far our chief effort has been to obtain evidence that would justify the use of the three methods mentioned, saturation with sodium chloride, dialysis and irradiation with ultraviolet rays. A more critical test of the value of the procedures we have followed is to compare all three of them when applied to the same sample. Our data are tabulated to show such a comparison in Table III.

Inspection of Table III shows that the quantity of globulin precipitated by the three methods agrees within reasonable limits. At a P_H value of approximately 6 all methods give about the same result. Dialysis consistently gives slightly lower results than the other methods, possibly for the reasons mentioned in an earlier paragraph.

Little need be said concerning the albumin determinations, for they obviously depend on the amount of protein estimated as globulin. As a matter of fact the chief value of determinations of albumin and non-protein nitrogen is as a check on the accuracy of the analytical procedures.

We have not made an intensive study of the method of heat separation but, as this procedure may prove useful, we are including a statement of our experience.

Aliquots of the sodium chloride extract (usually 25 cc.) were transferred to 50-cc. centrifuge tubes, then placed in a water-bath maintained at 49°. When the protein solution reached this temperature it was allowed to

TABLE III

A COMPARISON OF THE PROCEDURES USED FOR THE SEPARATION OF MUSCLE PROTEIN

		Method of separation				
		Saturation with NaCl		Irradiation,	Dialysis,	Heat and trichloroacetic acid, %
Date	Substance	PH 6, %[a]	PH 7, %	PH 6, %	%	
4/ 4/27	Globulin	61.8	57.8	54.7	68.7	80.8
	Albumin	20.5	25.1	17.1	12.4	
	Non-protein nitrogen	16.8		27.4	7.5	20.4
4/18/27	Globulin	59.4	57.7	55.7	56.0	82.1
	Albumin	23.9	24.6	16.9	21.3	
	Non-protein nitrogen	7.9	9.7	27.4	9.1	18.5
9/28/27	Globulin	61.4	57.8	53.8	43.5	84.5
	Albumin	27.1	25.6	29.9	22.0	
	Non-protein nitrogen	18.8	18.3	21.8	8.5	19.3
10/27/27	Globulin	59.3	50.1	42.3	46.1	83.5
	Albumin	28.3	30.8	43.5	20.9	
	Non-protein nitrogen	17.5	16.7	20.0	11.3	19.0
1/30/28	Globulin	51.3	51.3	57.3	52.3	78.0
	Albumin	23.5	20.1	21.3	16.9	
	Non-protein nitrogen	22.0	22.3	19.5	13.8	19.7

[a] All percentages are in terms of the extracted nitrogen.

remain until the precipitate was in a flocculent condition and the filtrate was clear. The time required varied from fifteen to twenty minutes. Our results are summarized in Table IV.

TABLE IV

SEPARATION OF MUSCLE PROTEINS BY HEAT COAGULATION

Date	2/24/24	4/23/24	5/21/24	7/2/24	7/12/24	7/18/24
Globulin	51.9	50.9	55.7	54.9	47.5	48.5
Albumin	28.3	16.1	25.3	18.4	19.6	22.0
Non-protein N	19.3	31.2	20.6	26.5	28.4	26.1
Date	7/28/24	10/5/24	10/9/24	11/22/24	1/28/28	2/13/25
Globulin	57.2	60.7	64.3	57.6	63.2	60.1
Albumin	19.5	22.2	25.6	33.3	18.7	28.9
Non-protein N	24.1	19.0	13.7	9.8	15.5	16.5

The ratio of globulin to albumin as estimated by our methods is uniformly about 2.5 to 1 and is not in agreement with most of the earlier reports. Thus Streyrer[7] and Saxl[8] both reported considerably more albumin than globulin. Mann[9] in his discussion of this subject says that myogen (albumin) makes up about 80% of muscle albumin. On the other hand, Stewart and Sollman[10] report that paramyosinogen (globulin) is more abundant than myosinogen (albumin).

At various times measurements were made of the hydrogen-ion concen-

[7] Steyrer, *Beitr. chem. Physiol. Path.*, **4**, 234 (1904).

[8] P. Saxl, *ibid.*, **9**, 1 (1907).

[9] Mann, "Chemistry of the Proteids," Macmillan and Co., London, **1906**, p. 389.

[10] Stewart and Sollman, *J. Physiol.*, **24**, 427 (1899).

tration of the filtrates from the globulin precipitates. We regard these measurements, Table V, as of some interest though they may have no immediate application to our problem.

TABLE V

CHANGE IN HYDROGEN-ION CONCENTRATION AFTER PRECIPITATION OF THE GLOBULIN

Initial *P*H of extract	4			5			6			7		
After saturation with NaCl	4.4	4.4	4.1	4.8	5.3	5.2	5.9	5.8	6.0	6.7	6.7	6.6
	4.3			4.6	5.1	4.8	5.7	5.7	5.7	6.5	6.4	6.7
				4.7	5.3		5.7	5.9		6.7		
After dialysis	7.4	7.9	7.5	7.4	7.8	7.1	7.5	7.9	7.5	7.6	7.2	7.9
	6.4	7.0	7.5	6.4	8.4	8.0	6.6	8.3	7.6	6.7	7.6	6.9
	6.3			6.7			6.9					
After irradiation with ultraviolet rays	Ten filtrates shifted from a *P*H of approximately 5.7 to a *P*H that varied between 6.8 and 7.											

When globulin was precipitated by saturation with sodium chloride, the *P*H values were shifted in some cases toward a point between 4 and 5, in others toward a point between 5 and 6. After dialysis the solutions were practically always more alkaline than the distilled water against which they were finally dialyzed.

Measurements of the hydrogen-ion concentration of irradiated solutions were not made unless the globulin was precipitated in a flocculent condition. In such cases the initial *P*H value was usually a little less than 6, and such solutions always become more alkaline. Our experience agrees with that of Clark[11] who noted that the *P*H of irradiated solutions increased. Mond[12] observed that the *P*H shifted toward the isoelectric point. Stedman and Mendel[13] observed that their solutions always became acid, but suggested that this shift was due to the effect on the solvent rather than to any change that occurred in the protein.

Summary

A 10% solution of sodium chloride has been used to extract the soluble proteins of muscle tissue. If the extract is kept near the freezing point the solubility of the proteins changes very slowly.

When the extract is saturated with sodium chloride, irradiated with a quartz mercury arc or dialyzed, a portion of the protein is precipitated, to approximately the same extent by each method. We have assumed that this precipitate is a globulin fraction. Our results indicate that this extract contains about 2.5 times as much globulin as albumin.

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[11] Clark, *Am. J. Physiol.*, **73**, 649 (1925).

[12] Mond, *Pflüger's arch. ges. Physiol.*, **200**, 374 (1923).

[13] Stedman and Mendel, *Am. J. Physiol.*, **77**, 199 (1926).