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Nitrogenous constituents of condensed milk, with special reference to total non-protein nitrogen, urea nitrogen and uric acid.

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NITROGENOUS CONSTITUENTS OF CONDENSED MILK,

WITH SPECIAL REFERENCE TO

TOTAL NON-PROTEIN NITROGEN,

UREA NITROGEN, AND URIC ACID.

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U N I V E R S I T Y   O F   L O U I S V I L L E

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by

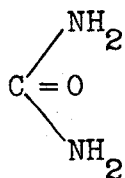Bess Burton Mathis.

Louisville, Kentucky.   June, 1920.

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The most important non-protein nitrogenous constituents of milk are urea, creatinine, creatine, and uric acid.

Urea is the diamide of carbonic acid

$$C \underset{NH_2}{\overset{NH_2}{<}} = O$$

and is the chief end-product, so far as nitrogen is concerned, of the physiological metabolism of the proteins of the foods and tissues. It has long been considered a matter of greatest importance to ascertain in what organ or tissues urea is formed. Investigations have gone so far as to demonstrate that it arises in part, at least, in the liver - that the liver cells are able to convert ammonium carbonate into urea which is then given to the blood and excreted by the kidney. It occurs in urine in relatively large quantities (two per cent), and is found also in slight quantities in other secretions - in milk, in traces, and in sweat. Urea is present normally in the blood in an amount equal to .028 per cent, and it has been shown that the tissues

generally contain urea in about the same concentration.

The history of the formation of urea in the body is not entirely known; but three sources of origin may be stated with some positiveness:

First, urea arises from ammonia salts which, in the liver, are converted into urea by a process equivalent to dehydration. The reaction may be represented as follows:

$$CO \begin{cases} ONH_4 \\ ONH_4 \end{cases} - 2H_2O \rightarrow CO \begin{cases} NH_2 \\ NH_2 \end{cases}$$
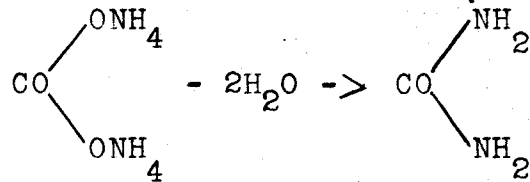
It would seem that the liver protects the body from the poisonous action of the ammonia compounds by converting them to urea, while in the same process, some of the CO_2 formed in the body is neutralized and prepared for excretion.

Second, urea arises from the mono-amino acids by a process of deaminization, whereby the NH_2 group is converted to ammonia and then probably to urea. There is evidence to show that this process of deaminization, as well as the further conversion of the ammonia to urea, takes place in the liver, although it is quite possible that both functions may be exhibited by other tissues as well.

On a large protein diet, the amount of
nitrogenous material supplied is in excess of the
amount needed for tissue construction. On this view
we can understand why the amount of urea eliminated
in the urine rises and falls with the amount of protein
taken as food. It may be supposed that the excess
nitrogen is promptly removed and excreted as urea. But
on the lowest protein diet or in starvation, when the
body is living on its own tissues, urea continues to be
formed, so that, in part, the urea of the urine repre-
sents nitrogen which has probably arisen from the destruc-
tion of protein tissue. The intermediate steps in this
latter process are not definitely known, but probably
they are analagous - that is, the protein passes through
the stage of amino acids and subsequently undergoes
deaminization.

Third, urea may arise from the arginine formed
in the cleavage of the protein molecule by conversion of
the contained guanidine radical. Unlike cases one and
two, the urea in this instance is formed from the
guanidine residue contained in the arginine and not from
the amino groups. Since arginine constitutes one of the
split products of the proteins during digestion, and
probably also one of the split products in the metabolism

of the protein of the tissues, there is reason to believe that part of the urea actually formed in the body arises by this method.

Urea has several quite important physiological actions. In the first place, it is a natural diuretic. Then tissues live much longer in the presence of some urea than in its absence. It has also a very definite function in the cells of some fishes, and possibly in mammalia also. It is one of the normal constituents of the cell and of the blood and other fluids of the body, and since these cells have for long years of time been selected to work with the highest degree of efficiency in this urea-containing medium, it is found that the addition of a little urea to artificial perfusion solutions is advantageous. The effect on mammalia is much less marked, since the amount of urea in the blood of mammals is very small, .02 to .04 per cent.

Urea is not, then, entirely inert.

Creatinine or methyl-guanidine-acetic acid anhydride

$$C \begin{cases} NH \text{———} CO \\ = NH \quad \Big| \\ N(CH_3) - CH_2 \end{cases}$$
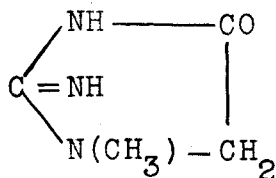
next to urea and the ammonia compounds, forms the
most important constituent of urine. It occurs also
in sweat, in milk, and in the muscles of fishes. Its
physiological history is imperfectly known. Under
constant conditions of life, the amount of creatinine
formed in the body is independent of the quantity of
protein eaten, and this fact indicates that it repre-
sents an end-product of the metabolism of living or
organized protein tissue rather than one of the results
of the metabolism of the food protein. But while the
creatinine excretion is independent of the protein of the
food, it is affected by the intake of creatine or creati-
nine. The ingestion of creatinine is followed by the
reappearance of most of it in the urine. Creatinine is
present in small amounts in the blood and in somewhat
larger amounts in muscular tissue.

Creatine or methyl-guanidine- acetic acid

$$C \begin{cases} NH_2 \\ = NH \\ N(CH_3).CH_2COOH \end{cases}$$
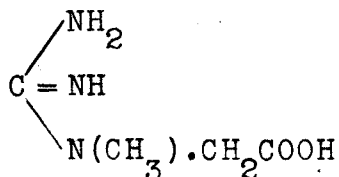
on the other hand, is present in muscular tissue to a
relatively large percentage, 0.5 to 0.6 grams per one
hundred grams of muscle, although Folin contends that

this large yield is due to post-mortem changes, and
that in living muscle little or none is present in free
condition. Creatine occurs in the blood in small
amounts, and in the urine of infants and children. It
also occurs under all conditions in which the body
either has no sugar to burn or is unable to burn it; in
starvation, diabetis, and sepsis. It occurs, too, under
circumstances of exaggerated wasting of muscle substance.

According to one view, the creatine and creatinine
are related and have a common physiological significance
in regard to the metabolism.

Creatine is regarded as an end-product of the
break-down of organized or living protein tissue. It is
produced constantly in the tissues and is normally con-
verted to creatinine before it is excreted in the urine.
Other observers believe that the metabolic history and
significance of creatine and creatinine are different.
The creatinine formed in the tissues represents an end-
product of the breaking down of the organized tissue
and, indeed, forms an index of the amount of this tissue
wear and tear, but it is given off to the blood and is
excreted in the urine as creatinine. The creatine, on
the contrary, while also constantly formed in the tissues
as a result of their metabolism is not converted to

creatinine, but undergoes some further and as yet
unknown metabolic change. The difficulty in accept-
ing the hypothesis of creatine destruction lies in the
fact that most of the nitrogen of the ingested creatine
does not appear in the urine, nor have Folin or others
found evidence to warrant accepting its transformation
into urea or ammonia. We can only come to the conclusion
that in such instances the creatine was being stored. On
this view, the significance of the creatine remains unde-
termined, and its genesis and fate are also left unsettled,
except so far as to deny its conversion to creatinine.

Steenbock and Gross are convinced that in one
form or another creatinuria is etiologically related to
protein metabolism, whether of exogenous or endogenous
origin, and that in addition, in a manner as yet unknown,
it is related to the creatine stored in the muscles and
other tissues as well. Reduced to its simplest terms,
the present immediate problem resolves itself into a
consideration of the mechanism of the formation of all
creatine. According to their hypothesis, creatine is an
end-product in the catabolism of certain precursors in
the protein molocule just as urea is of others. The
animal body has a fairly definite ability to destroy
creatine, and in creatinuria, creatine has been produced

in such amounts that the ability of the body to destroy
it has been exceeded.

Results of experiments by Gamble and Gold-
schmidt show that the infant differs radically from the
adult in its behavior toward ingested creatine - that
small amounts of ingested creatine lead to an increase
of the creatinuria. Results of other experiments per-
formed by them indicate that the creatine excretion of
infants bears a relation to the quantity of cow's milk
fed, and also other results indicate that the quantity
of whey given is more directly related to the degree of
creatinuria than is the total protein value of the food.
On the assumption that preformed creatine in milk
modifications is in proportion to the quantity of whey
present, the results suggest that the ingestion of
creatine is probably a large factor in the creatinuria
of infants fed on cow's milk.

There is no indication that creatine has any
specific role to perform in the animal body unless it
is that, by reversible reaction it can serve as a source
of guanidine grouping for arginine synthesis.

There are several possible sources of creatine
in the body, although the ultimate origin of it is uncer-
tain. It is related, on the one hand, to arginine from

which it might possibly be derived by oxidation and
methylation.   Creatine is a methylated amino acid,
a point of much interest since none of the amino acids
of the proteins are known to be methylated. On the
other hand, it may be regarded as an imidazole deriva-
tive. It is thus allied to histidine and to the purines,
both of which contain imidazole rings.

Uric acid, or 2,6,8, tri-oxypurine

$$
\begin{array}{ll}
\begin{array}{l}
H-N-C=O \\
\quad | \quad | \\
O=C \quad C-NH \\
\quad | \quad || \quad \diagup\!\!\!> C=O \\
H-N-C-NH
\end{array}
&
\begin{array}{l}
N=C-OH \\
\quad | \quad | \\
(2)HO-C \quad C-NH \\
\quad || \quad || \quad \diagup\!\!\!> C-OH(8) \\
N-C-N
\end{array}
\end{array}
$$
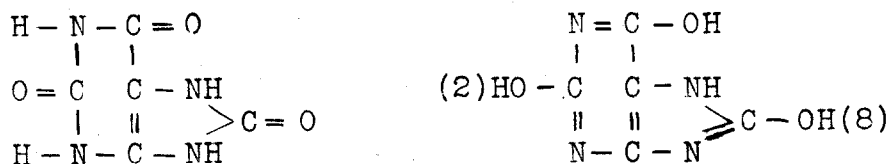
Lactam form.   Lactim form.

is of very particular interest because of its relation
to nuclear metabolism. It was long believed that uric
acid in urine was an intermediary product of protein
metabolism, which was usually almost completely
oxidized by the body to urea. However, we now know that
uric acid does not come from the ordinary protein metabo-
lism. The end-product of that metabolism is urea; but
it comes from the metabolism of the nucleins, both of
those of the food and those of the tissues.

The nucleins, it will be remembered, contain
nucleic acid, and nucleic acid contains purines, which
when oxidized, form uric acid.

There is no doubt that the ingestion of
nucleins increases the excretion of uric acid, and
the elimination of nucleins from the diet decreases the
excretion to a certain minimum, but does not abolish
it entirely. Therefore, some of the uric acid of the
urine has an exogenous source. But by cutting the
nucleins completely out of the food, or by starving,
it is not possible to suppress the uric acid excretion
entirely. This residual uric acid evidently must have
its origin either in the body tissues or else in the
bodies of the bacteria of the alimentary canal. Since
there is reasonfor thinking that the nuclei of the
body cells are undergoing metabolism it is generally
believed that this uric acid takes its origin, largely
at least, from the nucleins of the tissue nuclei. So
there is also uric acid of endogenous origin in the
urine.

There are in nearly all cells, and possibly
in all cells, autolytic or endocellular enzymes, or
nucleases, which decompose nucleic acid into its
various constituents. The various cleavages appear in
the autolysis of different cells and are believed to be
due to different nucleases. At any rate, the purine
bases are set free in most autolyses. It is, on the

whole, probable that some members of the group of en-
zymes are found in all tissues of the body, since the
partial conversion of guanine and adenine in their
nucleic acids to hypoxanthine and xanthine on autolysis,
appears to be a very common, if not a universal phenome-
non. In man, the uric acid, xanthine, and hypoxanthine
represent the final end-products of the metabolism of
nucleic acid, or, rather, of the purine nucleotide por-
tion of its molecule, and the extent to which they occur
in the urine indicates so much nucleic acid broken down.

Uric acid is but one of the purines. Purines
are important constituents of the most important constit-
uents of living matter, namely the chromatin of the cell
nuclei. The very pregnant question remains behind,
namely, can the animal organism make its purines from
other nitrogenous material of a non-protein kind, or
must it depend entirely on purine materials in the food?
Probably no nitrogenous substance in the urine has been
more studied than uric acid. Although present in small
quantities, it has long attracted attention because of
the problems involved in its origin, its significant
variation in disease, its insolubility, and above all,
because of its deposition in the joints in the form of
crystalline insoluble salts.

All of these non-protein nitrogenous compounds referred to, occur in small quantities in both cow's and human milk. Denis and Minot have found the amount of total non-protein nitrogen in cow's milk to be about 22.9 mg. per 100 cc., and of this total, 9.3 mg. occur as urea nitrogen, 4.3 mg. occur as creatinine, 2.4 mg. are present as creatine, while 1.5 mg. are in the form of uric acid. The total non-protein nitrogen in human milk amounts to about 33 mg. per 100 cc., of which 12.6 mg. are found as urea nitrogen, 1.5 mg. occur as creatinine, 3.5 mg. are in the form of creatine, and 2.5 mg. are present as uric acid.

Whether these nitrogenous substances present in milk and in other foods are in any way responsible for the non-protein nitrogenous constituents found in urine is as yet unknown, although such a relationship would seem highly probable.

It has been the object of my experiments to determine whether or not the process of condensing cow's milk in any way changes the amounts of total non-protein nitrogen, urea nitrogen, or uric acid present in the milk.

The three brands of condensed milk, Pet, Carnation, and Wilson, were used in the experiments, and

the methods employed were those described by Denis

and Minot in the Journal of Biological Chemistry,

March, 1919, pages 355, 357, and 364.

No difficulty was encountered in the applica-

tion of the methods for the determination of urea nitro-

gen and uric acid, but a slight modification was necessary

in the case of total nitrogen. When the milk filtrate,

freed from protein, carbohydrate, and fat, and digested

with the acid digestion mixture, was diluted to a volume

of 25 - 50 cc., it was found that it could not be

successfully Nesslerized. The difficulty seemed to be

in the concentration. The method finally adopted was

a modification of that of Folin and Wu. It is as

follows: Take 20 cc. of protein-free milk filtrate

and introduce it into a large test tube. Add to this

1 cc. of acid digestion mixture (see 1. below) and a

small pebble. Heat this tube with its contents over a

low flame to boiling, continue to boil until the dark

mixture turns water white, allow the tube to cool and

make the volume up to 100 cc. in a volumetric flask.

From this solution draw out 25 cc. and Nesslerize with

15 cc. of Nessler's solution. The standard is prepared

as follows: Introduce 1.5 cc. of ammonium sulphate

(1 cc. = 22.949 mg. nitrogen) into a 100 cc. volumetric

flask, and add 1 cc. of the acid digestion mixture.
Make this up to 100 cc. with distilled water. Draw out
25 cc. and Nesslerize with 15 cc. of Nessler's solution.

(1) The acid digestion mixture is made by
taking 100 cc. of 85% phosphoric acid, 300 cc. of
concentrated sulphuric acid, and 15 cc. of a 10% copper
sulphate solution. Mix these thoroughly.

At the beginning of each determination the
condensed milk was diluted with distilled water until
the specific gravity was the same as that of cow's
milk.

Practically the same difficulty was encountered
in the determination of non-protein nitrogen in blood
by the method of Folin and Denis. The acid digestion
mixture recommended by Denis and Minot was found to give
better results than that described by Folin and Wu.
in their modification of the method of Folin and Denis.
The procedure is as follows: Take 5 cc. of protein-
free blood filtrate and introduce into a large test tube.
Add to this 1 cc. of acid digestion mixture (see 1.
below) and a small pebble. Heat this tube with its
contents over a low flame to boiling, continue to boil
until the dark mixture turns water white, allow the tube
to cool and make the volume up to 100 cc. in a volumetric

flask, with distilled water. From this solution draw
out 25 cc. and Nesslerize with 15 cc. of Nessler's solution.

(1) The acid digestion mixture is that described
by Denis and Minot in the Journal of Biological Chemistry,
March, 1919, page 356. The standard is prepared as
follows: Introduce 1 cc. of ammonium sulphate (1 cc.=
0.3 mg. nitrogen) into a 100 cc. volumetric flask, and
add 1 cc. of the acid digestion mixture. Make this up
to 100 cc. with distilled water. Draw out 25 cc. and
Nesslerize with 15 cc. of Nessler's solution.

A series of experiments on Pet, Carnation,
and Wilson brands of condensed milk with regard to the
amounts of total non-protein nitrogen, urea nitrogen,
and uric acid present showed the following results:

Pet   (mg. per 100 cc. of milk)

Total Non-Protein Nitrogen	Urea	Uric Acid
20.36	14.60	0.34
20.57	14.00	0.35
25.45	13.90	0.31
21.55	13.76	0.35
21.15	14.15	0.30
20.39	14.54	0.33

Carnation (mg. per 100 cc. of milk)

Total Non-Protein Nitrogen	Urea	Uric Acid
28.63	11.8	0.32
30.54	13.1	0.32
26.51	13.3	0.31
29.39	12.6	0.32
29.77	11.9	0.30
29.20	13.1	0.32

Wilson (mg. per 100 cc. of milk)

Total Non-Protein Nitrogen	Urea	Uric Acid
22.48	13.8	0.36
20.78	15.6	0.35
19.72	16.3	0.32
24.94	14.8	0.30
22.16	15.1	0.32
21.32	14.9	0.31

These results indicate that the amount of
total non-protein nitrogen present in condensed milk
is practically the same as that recovered from cow's
milk by the methods of Denis and Minot. The quantity
of urea in condensed milk, however, is considerably
greater than that in the uncondensed milk, the former,
in some instances, showing an increase of from one
third to one half in urea nitrogen. At the same time,
the amount of uric acid recovered shows a marked
decrease as compared with cow's milk, about one fourth
as much uric acid being present after the process of
condensing. Due to the fact that such conditions
exist, it is probable that an internal molecular
-rearrangement of the nitrogenous constituents of the
milk has taken place -- that in the process of evapora-
tion some of the uric acid and other purine bases have
been converted into urea. There cannot have been, from
our results, any loss in the nitrogenous constituents, in
that the total non-protein nitrogen remained constant. The
rearrangement must have taken place in the breaking down
of the uric acid. The results obtained point distinctly
to a rearrangement of the nitrogenous constituents.

* * *   B I B L I O G R A P H Y   * * *

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