TRANSLATION NO. 2197

DATE: 30 April 1962

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

17

T-623-2

LUMINESCENCE METHOD FOR THE DETERMINATION OF PROTEIN IN MILK

[Following is the translation of an article by S. Konev, T. I. Lyskova, and P. N. Saloshenko, Laboratory of Biophysics and Isotopes, AN BSSR, Minsk, published in the Russian-language periodical Biofizika (Biophysics) No 8, 1963, pages 260-268. It was submitted on 30 Apr 1962.]

In 1958 one of us jointly with I. I. Kozunin proposed a luminescence express-method for the determination of protein in milk and a device for carrying it out [1-3]. This method makes it possible to lessen the cost of the determination process and to speed it up by several dozen times.

The principle of the method is based on the measurement of integral intensity of fluorescence of tryptophan from proteins in milk which has been diluted by 10 times with water following excitation with light of a wave length of 250-290 nm. The intensity of the light which stimulates the luminescence of protein was equalized during each measurement according to a standard screen. Thanks to this the intensity of protein luminescence did not depend on fluctuations in the intensity of exciting light and, as was proposed, is connected in a range of specific dilutions by a direct proportional dependence with the content of protein in milk. This proposal is based on the linear nature of the dependence of the intensity of luminescence of milk on the dilution (beginning with dilutions of 1:10 with a layer thickness of 1 mm), the disappearance of luminescence after the settling of proteins out of the milk, and the conformity in the results of determining the content of protein in various samples of milk by the Kjeldahl method and by the proposed method (18 samples).

However, further tests showed that in individual samples quite significant divergences (up to 0.4% of absolute protein content) are observed between the luminescence method and the Kjeldahl method. We observed similar divergences during experiments on a test model of a device, produced at the Laboratory of Biophysics and Isotopes, AN BSSR on order of the Administration for the Meat and Dairy Industry, Council of the National Economy, BSSR, and also at the All-Union Institute of Animal Husbandry with the adoption of an FPM-1 device (photo and transparency meter) produced at the instrument making plant for the Ministry of Agriculture (MSKh) of the USSR.

The fluorescence method recently became an object of special investigation on the part of the Dutch investigators [4]. Koops and Wijnand confirmed our data on the conformity of results in the determination of protein by the luminescence method and by the Kjeldahl method for individual samples of milk from different cows (15 samples).

They calculated the correlation coefficients between the content of protein based on Kjeldahl and on the luminescence method for our /1/ (0.9785) and their (0.9647) data and showed their high degree of conformance. At the same time the reliability limit of the method (for 95%), based on their calculations, should comprise wholes of ± 0.2%. In two tests out of fifteen Koops and Wijnand observed divergences from the data of the Kjeldahl method on an order of 0.3%.

The stated unit divergencies between protein content determined by Kjeldahl and by the intensity of luminescence compel a more detailed analysis of the possible causes of their origination.

First of all it is necessary to note that luminescence analysis of protein in milk differs from the luminescence analysis of other substances by a whole number of peculiarities. Without even speaking of the fact that luminescence intensity has to be measured in a turbid heterogeneous medium, it is possible to point out four more factors which are capable of disrupting the direct proportionality between protein content and intensity of luminescence: 1) nonactive screening absorption and scattering of exciting light and the light of luminescence in milk; 2) luminescence of nonprotein centers; 3) variability of quantum yield of tryptophan in proteins; 4) inconstancy of the ratio of weight of tryptophan to weight of protein in the sample. The latter is connected with the fact that in contrast to other comparatively low molecular substances in protein a component luminesces which does not specify the quantity by weight of the substance. The entire "heavy" macromolecule does not luminesce, but only some of its "light" components, primarily tryptophan fragments, the number of which even in a molecule of an individual protein can experience some variations.

In connection with what is said in this paper the task is laid down to attempt to appraise the extent of the influence of the above stated factors on the accuracy of luminescence analysis and to note possible ways of augmenting it.

Method

In the tests milk was used which was taken from mixed samples (can) from the Minsk Dairy Plant. Dilution was carried out (1:10) with distilled water or an 8 M solution of urea. Intensity of luminescence was measured in passing light partially on a device which differed from the previously described one /3/ by replacement of the FSK-M-1 photoresistor with a FEU-26 photomultiplier and feeding the photomultiplier and BUV-15 bactericidal uriol lamp from a storage battery through a voltage step-up converter, and partially on a unit made up of a SF-4 spectrophotometer, FEU-18 photomultiplier, "Kaktus" type direct current amplifier or pip amplifier, and a scaling unit.

Measurement of the luminescence spectra of milk depending on the wavelength of exciting light was performed on a unit made up of two monochromators (DMR-1 and a monochromator with diffraction grating)

and an EPP-09 recording potentiometer kindly given to us by G. P. Gurinnovich (Institute of Physics, AN BSSR) to whom the authors express their deep thanks.

Results and a Discussion of Them

There is no doubt that the first two factors of errors (nonactive absorption and scattering of exciting light and the light of luminescence, and also luminescence of nonprotein centers) theoretically should influence the intensity of protein luminescence of milk. However, since in the area of 250-290 nm fats and carbohydrates do not have any significant absorption and other organic substances are present in negligible quantities in comparison with protein, the practically nonactive absorption in this case can be disregarded.

Actually in Figure 1 are given the spectra of excitation of fluorescence for milk (area of fluorescence registration 320-400 nm) which has been diluted with water and urea. It is clear from the spectra that they are very similar with the spectra of excitation of pure protein solutions [6, 7]. No distortions are revealed in the spectra which would indicate a stratification of effects of nonactive absorption and scattering (additional minimums) or, conversely, active absorption by other luminescent centers except tryptophan (additional maximums) in the excitation spectra.

Moreover, if one were to consider the linear nature of the dependence of luminescence intensity on the dilution of the milk and the attenuation of it by more than two orders after the settling of proteins, then the error which develops as a result of the action of the first two factors can be considered insignificant for this method.

Convincing arguments in favor of the primarily protein nature of luminescence are the fact of the very close conformity of luminescence spectra of milk which are monochromatically excitable with light of 280 nm (maximum of protein absorption) and 250 nm (minimum of protein abosrption), and also the similarity of polarization spectrum of milk luminescence, which has a maximum at 270 nm (+12%) and minimums at 240 (+2%) and 282 (+8%), with the polarization spectra of tryptophan and proteins [8].

A more complex problem is the degree of influence on the accuracy of quantitative analysis which is exerted by the variability of quantum yield of fluorescence by tryptophan in proteins. The quantum yield of fluorescence by tryptophan in milk may be influenced by four various factors: 1) variations in the relationship of protein fractions (casein, albumins, globulins, etc.); 2) difference in the physico-chemical state of protein in milk, for example, in pH, in the degree of aggregation, etc.; 3) presence of various extinguishers of luminescence; 4) processes of intertryptophan migration of energy between neighboring macromolecules under conditions of dense packing of the granule.

The problem is solved most simply for concentration quenching if it is considered that fluorescence of proteins in milk is depolarized no greater than, for example, in a strongly diluted solution of albumin, blood serum, or casein (+ 12% at 270 nm). Since the effects of concentration quenching of tryptophan fluorescence (if in general they exist in protein) are observed after a lessening of polarization, then the absence of depolarization of protein luminescence of milk may be viewed as proof of the insignificance of the influence of concentration effects.

It would hardly be justified to assume also that milk usually contains some extraneous quenchers of tryptophan luminescence, in any case in quantities which would change its quantum yield by more than 2%. In our tests dialysis of ten samples against saline solution which was equivalent to milk serum did not change the intensity of luminescence, although by this all the ingredients of a nonpolymer nature should have been removed.

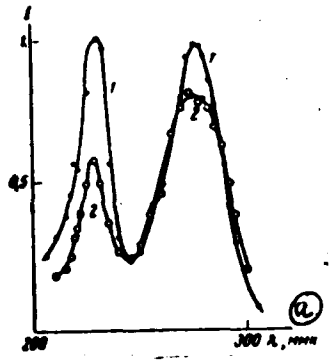

Fig. 1. Spectra of excitation of milk fluorescence (5 mm layer) in a dilution of 1:50.
1 - distilled water; 2 - 8 M of urea.
Key: (a) nm.

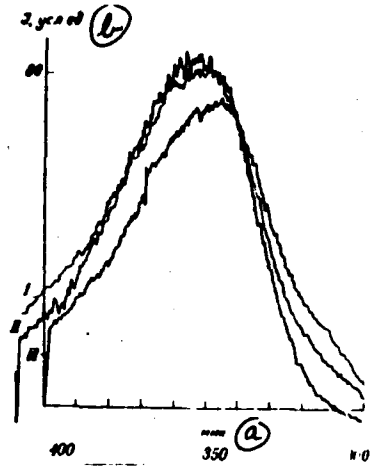

Fig. 2. Spectra of fluorescence of milk in a dilution of 1:100.
I - in urea with excitation on a wave length of λ 250 nm; II - in urea with excitation on a wave length of λ 280 nm; III - in water with excitation on a wave length of λ 280 nm. Corrections for spectral sensitivity of the unit, light scattering, and reabsorption are not inserted.
Key: (a) nm; (b) conditional units.

Somewhat different is the affair relative to the first two factors which are capable of exerting an influence on the quantum yield of tryptophan fluorescence. It is known, however, that although the greatest majority of milk proteins (80%) belong to the casein fraction [9, 10], still the percentage distribution based on various protein fractions may experience certain individual variability [10]. On the other hand the quantum yield of tryptophan luminescence is not the same in different proteins [6]. From here a change in the percentage ratio between quantities of different protein fractions in milk can lead to a change in the distribution of tryptophan between these fractions and, as a result of this, to a change in the quantum yield of all the tryptophan found in the milk. As a result of this sometimes more and sometimes less tryptophan may turn out to be in a fraction of protein in which the tryptophan possesses, for example, a higher quantum yield. Therefore different intensities of luminescence may correspond to the same content of tryptophan in two different samples. There is also the complete possibility of the influence of the physicochemical state since it has been proven that casein is found in milk in the form of granules with a molecular weight on an order of several hundreds of millions [9, 11]. Here variations in amount or density of packing of the protein in the granules may influence the quantum yield of luminescence, since not only quantum yield but also the spectrum of luminescence of aromatic amino acids depends on their physical state of aggregation. It is sufficient to point out that the spectrum of emission of tryptophan and its quantum yield are different in solution and in crystals [12, 13], in native and denatured protein [14, 15], and in a solution of protein and in a film [15, 16]. Cases have been described of an increase in ultraviolet luminescence of cells at the moment of mitosis [17], which is also probably connected with differences in the physicochemical state of the proteins.

For clearing up the interconnection between the physical state of aggregation of proteins in milk and their luminescence it was first necessary to find a method for influencing the milk, selectively changing only the supramolecular structurality of proteins. A realistic approach to this were data in the literature that urea leads to the breakdown of large granules of casein into a monodisperse [9] state (electrophoretic and turbidimetric data [9]). Here the differences in the position of the maxima of fluorescence spectra and its quantum yield in various proteins are smoothed out [6]. Therefore it seemed probable to expect that processes of disaggregation and denaturing should bring the physicochemical state as if to the same level, to standardize it and by this eliminate its influence on quantum yield and fluorescence spectrum of tryptophan. At the same time it should also lessen the influence of the affinity of tryptophan to various protein fractions.

In the first tests on the dilution of milk in an 8 M solution of urea it was revealed that it exerts a certain, but very insignificant, influence on the structure of the electron-fluctuation levels of tryptophan remnants both in the basic and the singlet excited states.

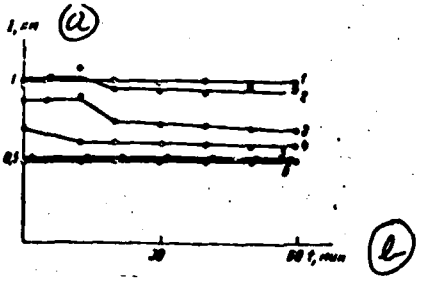

Fig. 3. Dependence of relative quantum yield of luminescence of milk; dilution 1:10.
1 - dilution with distilled water; 2 - 1 M urea; 3 - 2 M urea; 4 - 4 M urea; 5 - 6 M urea; 6 - 8 M urea.
Key: (a) I, lm; (b) t, min.

It can be seen from Figures 1 and 2 that urea causes a very small widening of the spectra of fluorescence and the spectra of excitation of fluorescence. Here the maximum of the spectrum of protein fluorescence shifts from 335 to 345 nm (with the introduction of a correction for the spectral sensitivity of the unit, but without a correction for light scattering and reabsorption). These insignificant spectral changes are also observed in solutions of individual proteins and may be explained by the denaturing action of urea and a change in the properties of the molecular environment of tryptophan.

Of considerably great importance in the light of the problem which interests us is the capacity of urea to approximately cut in half the quantum yield of the protein fluorescence of milk. A whole number of considerations compel the assumption that processes of disaggregation of proteins, and not their denaturation, are most important in the mechanism of decreasing quantum yield.

First of all, as can be seen from Fig. 2 the decrease in quantum yield with the increase in the concentration of urea does not take place abruptly upon reaching a specific threshold, as this would be natural for the denaturing mechanism, but gradually in complete accordance with the gradualness of process of disaggregation [9].

Already concentrations of urea which do not lead to denaturation of protein (1.0 and 2.0 M solutions) nevertheless cause a decrease in quantum yield which develops in time. At the same time passage over the threshold of denaturing changes, that is, transition from 2 M to 4 M and more saturated urea, is not connected with a sharp abrupt drop in quantum yield. (Fig. 3)

It is natural that thermal denaturing of milk proteins (boiling), which does not lead to essential changes in turbidity (disaggregation), also does not exert an influence on the quantum yield of milk which has been diluted in water or in urea (Table 1).

---

[1] Virtually they determined the integral intensity of luminescence of a 5 mm layer of milk (1:20) in the region of 320-400 nm after transformation with the help of a concentrated solution of fluorescein with a fluorescence spectrum in the yellow-green area of the spectrum for eliminating the influences of the spectral sensitivity of the photocathode and changes in the contour of the fluorescence band of tryptophan, taking into consideration that under the action of urea the absorption spectrum of proteins essentially is not changed /18/.

On the other hand, for the most part disaggregating influences (ultrasonic vibrations) lead to a strong drop in quantum yield which varies little even after dilution in 8 M urea. The influence of an aggregate condition of protein in milk on the intensity of luminescence of its tryptophan is also testified to by data from Koops and Wijnand /4/, who observed a reduction in the time of intensity of luminescence in milk which had been diluted with water (1:500) and a stabilization of this process with 0.01 M $CaCl_2$ which impedes the process of disaggregation /4, 9/.

What has been stated above makes it possible to draw three conclusions which are important to us: 1) quantum yield of fluorescence depends on the degree (or nature) of aggregation of proteins in milk; 2) dissolving of milk in 8 M urea leads to uniform disaggregation; 3) based on the extent of drop in quantum yield following dissolving in urea it is possible to judge the level of the state of aggregation of proteins in the original samples of milk.

It can be seen from the data for ten aggregate samples which are presented in table 2 that the reduction of relative quantum yield of luminescence after the influence of urea is by no means the same and fluctuates within limits of 2.2 to 1.7 times. These differences in milk samples in response to the same influence apparently testify to the individual variability in the dimensions of protein granules or to the nature of protein packing in them in different animals. Further tests showed that namely these differences are capable of exerting the most significant influence on the quantum yield of fluorescence of milk proteins, and through it on the accuracy of analysis.

This is clearly manifested in tests comparing the intensity of protein luminescence in aggregate samples of milk, diluted with water and with urea, among themselves and with the results from determining protein content by the Kjeldahl method (Tables 3 and 4). Analysis of protein by Kjeldahl determination in one test was carried out in the dairy laboratory at VIZH /All-Union Scientific Research Institute of Livestock Breeding/ and in the other in the chemistry lab at the VNIMI /All-Union Scientific Research Institute of the Dairy Industry/ by K. K. Markova and P. F. Dyachenko, for which the authors take this time to express their deep gratefulness.

Table 1

Influence of ultrasonic vibrations and thermal denaturing on the intensity of protein luminescence in milk

Молоко в разведении 1 : 10 в различных состояниях	Интенсивность люминесценции		
	в воде	в 1 М мочевине	в 8 М мочевине
1 — свежее	100	98	51
2 — после кипячения	99	—	50,5
3 — после воздействия ультразвуком (4,5 кгц, 3 часа)	—	60	51
4 — контроль после трехчасовой экспозиции разведенного молока без ультразвука	—	95	—

Key: (a) Milk in a dilution of 1:10 in various conditions; (b) Intensity of luminescence; (c) in water; (d) in 1 M urea; (e) in 8 M urea. 1 - fresh; 2 - after boiling; 3 - after the influence of ultrasonic vibrations (4.5 kHz, 3 hrs); 4 - control after 3-hour exposure of diluted milk without ultrasonic vibrations.

Table 2

Influence of 8 M urea on the relative quantum yield of protein luminescence in milk (dilution 1:20)

№ пробы	Интенсивность люминесценции, имп. 10 сек.		После нормировки на %	
	в воде	в 8 М мочевине	в воде	в 8 М мочевине
1	36 470	17 900	100	49,1
2	38 100	21 500	100	56,5
3	43 460	19 550	100	45,0
4	39 170	20 180	100	51,5
5	37 570	19 780	100	52,5
6	36 560	20 450	100	55,1
7	37 700	20 660	100	54,8
8	42 200	23 700	100	56,1
9	40 400	24 380	100	60,3
10	28 540	15 310	100	53,6
Среднее	38 057	20 330	100	53,4

Key: (a) No. of sample; (b) Intensity of luminescence, imp/10 sec; (c) in water; (d) in 8 M urea; (e) After normalization; (f) Average.

It can be seen from the tables given that in individual samples (Table 3, samples 1, 4, 6, and 7; Table 4, sample 8) the divergence between intensity of fluorescence and protein content is reduced considerably after the addition of urea. This speaks directly for the supramolecular, aggregation nature of errors in fluorescence analysis in these samples.

After dilution of milk with urea the average absolute divergence between luminescence analysis and Kjeldahl determination is essentially reduced. Thus the average absolute divergence in the first test comprised 0.068% protein during dilution in urea and 0.14% during dilution in water (relative divergences of 2.25 and 4.6% correspondingly).

In the second test the data of luminescence analysis in urea deviated from the data of Kjeldahl determination on an average by 0.085%, while luminescence analysis in water produced a divergence in 0.14% (relative errors of 2.9 and 4.8% respectively). The average absolute divergence based on the two tests for milk diluted with urea comprised all told 0.07%. The correlation coefficient, calculated by the formula

$$r = \frac{\Sigma (x - \bar{x}) \Sigma (y - \bar{y})}{\sqrt{\Sigma (x - \bar{x})^2 \Sigma (y - \bar{y})^2}}$$

for orders of intensity of luminescence in water [nitrogen based on Kjeldahl (1) and intensity of luminescence in urea - nitrogen based on Kjeldahl (2)] comprised 0.9500 (1) and 0.9748 (2) correspondingly for the first test, and 0.9406 (1) and 0.9478 (2) for the second test (Table 5). On the average the correlation coefficients were 0.9475 for water and 0.9691 for urea. There is no doubt in the authenticity of the correlation coefficients.

On the basis of correlation coefficients based on the formula

$$t \cdot S_r = 2.12 \sqrt{\frac{\Sigma (x - \bar{x})(1 - r^2)}{n - 2}}$$

(for 95% level of significance) we calculated the authenticity limits of divergence with the Kjeldahl method (Table 5), which turned out to be somewhat less in comparisom with the spectrophotometric method ($\pm$ 0.16) [19] and approximately identical with the method of determining sorbtion of the dye ($\pm$ 0.12) [20].

Although differences between correlation coefficients of 0.9475 and 0.9691 are not reliable (based on the Fischer transformation)

$$t = \frac{z_1 - z_2}{Sd_z} = 0.85$$

apparently in a large number of samples, especially individual ones,

increasing the accuracy, then the action of urea should all the same be expressed.

The use of 8 M urea as the solvent , in addition to increasing the accuracy of the method, involves a certain number of purely practical advantages.

Since the quantum yield following the influence of 8 M urea drops exceedingly fast and subsequently remains constant for a prolonged period of time (Fig. 2), then the determination of protein content in milk can be carried out immediately after its dilution.

Table 3

Results of determining protein in aggregate samples of milk based on the Kjeldahl and luminescence methods (Dairy Laboratory, VIZH)

(a) No. пробы	(b) % белка по люминесцентному способу: (c) в воде	(d) в мочевине	(e) % белка по Кьельдалю	(f) Абсолютное расхождение: (g) между графами 3 и 1	(h) между графами 3 и 2
	1	2	3		
1	2,9	3,0	3,11	−0,21	−0,11
2	3,35	3,23	3,35	0,00	−0,12
3	2,95	2,90	2,86	+0,09	+0,04
4	3,09	2,94	2,88	+0,21	+0,06
5	2,95	2,98	3,09	−0,14	−0,11
6	3,40	3,07	3,04	+0,36	+0,03
7	3,28	2,93	2,94	+0,34	−0,01
8	3,00	2,97	2,93	+0,07	+0,04
9	2,95	3,11	3,00	−0,05	+0,11
10	3,00	2,98	2,91	+0,09	+0,07
11	3,13	3,10	3,18	−0,05	−0,08
12	2,90	3,10	3,06	−0,16	+0,04
(i) Среднее	3,07	3,025	3,03	0,14	0,008

Key: (a) No. of sample; (b) % protein based on luminescence method; (c) in water; (d) in urea; (e) % protein by Kjeldahl; (f) Absolute divergence; (g) between columns 3 and 1; (h) between columns 3 and 2; (i) Average.

Along with this, samples of formalin fixed milk which are repeatedly diluted with urea on the 4th, 6th, and 8th days of storage show the same intensity of luminescence in comparison with the first day, while samples diluted with water already on the 5th day reveal noticeable differences (Table 4). Based on this it can be assumed that the use of urea makes it possible to determine the content of protein even in samples of milk which prior to analysis were stored for quite a long time. Judging by certain data [21], the use of urea makes quantum yield less dependent on those differences in the physicochemical state of protein which are connected with fluctuations of the pH of milk which are possible in practice.

Table 4

Results in the determination of protein in aggregate milk samples based on the Kjeldahl (Chemical Laboratory, VNIMI) and luminescence methods

(a) № проб	(b) Люминесценция в молоке							(c) % белка по Кьельдалю	(d) Абсолютное расхождение между гр. 9 и 8	(e) Люминесценция в воде			(f) Среднее между гр. 11 и 12	(g) Абсолютное расхождение между гр. 9 и 14
	5.1.62	6.1.62	9.1.62	11.1.62	13.1.62	15.1.62	среднее			5.1.62	6.1.62	10.1.62		
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	2,99	2,99	—	2,99	2,99	2,99	2,99	2,99	0,00	2,99	2,99	2,99	2,99	0,00
2	3,22	3,16	3,20	3,38	3,16	3,16	3,21	3,20	−0,01	3,33	3,34	3,61	3,33	+0,13
3	2,86	2,83	2,6.	2,79	2,76	2,72	2,76	2,57	−0,19	2,75	2,70	3,20	2,73	+0,16
4	2,84	2,84	2,87	2,86	2,80	2,75	2,82	2,82	0,00	2,94	2,80	3,20	2,87	+0,05
5	2,80	2,78	2,83	3,08	3,04	2,91	2,92	2,70	+0,22	2,94	2,96	2,78	2,95	+0,25
6	2,93	2,89	3,04	2,99	2,94	2,94	2,96	2,99	−0,03	3,01	2,89	3,57	2,95	−0,04
7	2,88	2,80	2,84	2,99	2,87	2,82	2,85	2,99	−0,14	2,94	2,73	3,36	2,84	−0,17
8	2,88	2,84	2,64	2,86	2,77	2,69	2,76	2,86	−0,10	2,66	2,64	3,08	2,65	−0,21
(h) 9	3,01	—	3,02	2,99	2,93	2,75	2,94	3,02	−0,08	2,94	2,85	3,34	2,89	−0,13
Среднее	2,943	2,89	2,89	2,98	2,91	2,86	2,91	2,904	0,085	2,94	2,88	3,23	2,907	0,14

Key: (a) No. of sample; (b) Luminescence in urea; (c) % protein based on Kjeldahl; (d) Absolute divergence between columns 9 and 8; (e) Luminescence in water; (f) Average between columns 11 and 12; (g) Absolute divergence between columns 9 and 14; (h) Average.

Finally it is possible to point out still one more advantage, though it is less significant, connected with the use of urea. Solutions of milk in urea possess relatively less diffusion of light, as a result of which the linear dependence of intensity of luminescence - dilution of milk begins to be realized somewhat earlier than in aqueous solutions (with a milk layer thickness of 1.0 mm at dilutions of 1:8 instead of 1:10).

Thus the use of urea should to a considerable degree increase the accuracy and stability of the results of luminescence analysis as a result of a whole number of factors, the basis of which is the "standardization" of quantum yield.

In viewing the possible factors of the lessening of accuracy in luminescence analysis up until now we have left aside the problem of the degree of constancy in the ratio of weight of tryptophan to the weight of protein in milk samples from different cows (differences in breeds, phases of lactation, age, feeding), though this plays a resolving role for the quantitative method.

A direct answer to this problem would have been a comparison of content of tryptophan and protein in different samples of milk. Unfortunately we were not able to find any references to such investigations in the literature and we were still not able to achieve

a degree of accuracy in the quantitative determination of tryptophan in milk (± 2-3%) which was sufficient for calculations. Therefore for solving the problem it is necessary to resort to an indirect argument. First of all attention is drawn to the fact that, based on the data of Haugaard and Dam [19] the content of protein, determined by absorption at 290 nm in alkalized and skim milk for 43 samples conformed with the results of Kjeldahl determination. For the 95% level of significance the reliable limits of divergence between methods comprised ± 0.16 and the correlation coefficient between results was exceedingly high (0.9804). Since in alkalized milk at 290 nm absorption pertains to tryptophan and tyrosine, then it is apparent that the content by weight of protein, determined based on nitrogen, is closely connected with the content by weight of tryptophan and tyrosine determined by absorption.

Table 5

Variation-statistical processing of the results of the tests

(a) Опыт	Число наблюдений (b)	$r = \frac{\Sigma(x-\bar{x})(y-\bar{y})}{\sqrt{\Sigma(x-\bar{x})^2\Sigma(y-\bar{y})^2}}$		$t = \frac{r\sqrt{n-2}}{1-r^2}$	$t \cdot S_r \to \pm 2{,}12\sqrt{\frac{\Sigma(x-\bar{x})(1-r^2)}{n-2}}$	
		в воде (c)	в мочевине (d)	в воде (c)	в мочевине (d)	в воде (c)	в мочевине (d)
1. Определение по Кьельдалю (ВИЖ)	12	0,9500	0,9748	30,4	43,6	±0,1	±0,07
2. Определение по Кьельдалю (ВНИМИ)	9	0,9406	0,9478	19,1	20,7	±0,15	±0,14
3. В целом по двум опытам	21	0,9475	0,9691	46,3	66,9	±0,12	±0,09

Key: (a) Test; (b) Number of observations; (c) in water; (d) in urea; 1 - Kjeldahl determination (VIZH); 2 - Kjeldahl determination (VNIMI); 3 - All together by the two tests.

In our tests on aggregate samples of milk a comparison was made of the intensity of fluorescence with the optical density at 280 nm of the same samples of milk in 95% glycerin after introduction of the correction for diffusion of light by extrapolation from the region of 450-350 nm (Table 6).

First of all in the case of comparison with the Kjeldahl method a completely satisfactory conformity is noted between the intensity of luminescence in urea and optical densities and a considerably worse conformity for the intensity of luminescence in water.

Secondly the results cited by us in Tables 3 and 4 on the intensity of luminescence of milk in urea (with "standardized" quantum yield of tryptophan fluorescence) show such a similarity with the Kjeldahl method which would not take place in the event of variability in the ratio of weight of tryptophan to weight of protein.

In reality since the maximum relative error of the Kjeldahl method comprises a value on the order of 3% * and it is approximately that for the maximum error in the process of fluorescence measurement, it can be expected that the relative divergence between the two methods will not exceed 6%, i. e., with a 3.0% content of protein in milk the divergence $\leq$ 0.18% of absolute protein content. It can be seen from Tables 3 and 4 that in practice the maximum absolute divergence between luminescence and chemical analyses does not exceed 0.2%.

---

* A parallel determination of nitrogen by the Kjeldahl method in our laboratory yielded an average absolute divergence with the Kjeldahl method at the VNIMI of 0.1%.

Table 6

Connection between content of protein based on intensity of luminescence and optical density at λ=280 nm

(a) № пробы	(b) По интенсивности люминесценции		(e) По оптической плотности при 280 мкм, разведение 1:50 в глицерине
	(c) в воде	(d) в мочевине	
1	3,22	3,22	3,22
2	3,12	3,08	3,12
3	2,74	2,90	2,87
4	2,98	3,05	2,91
5	2,53	3,03	3,07

Key: (a) No. of sample; (b) Based on intensity of luminescence; (c) in water; (d) in urea; (e) Based on optical density at 280 nm, dilution of 1:50 in glycerin.

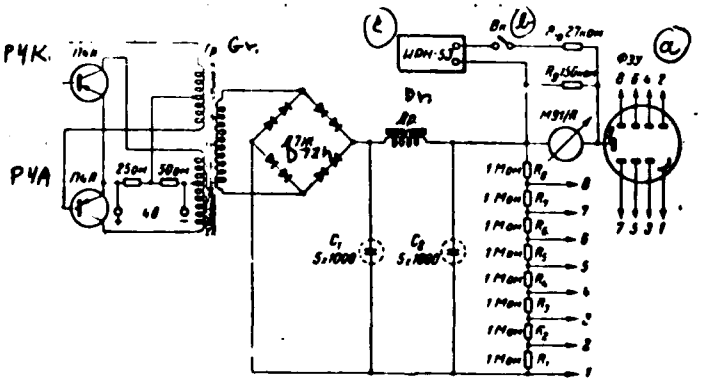

Fig. 4. Electrical circuit for the power supply of the photomultiplier during measurement of intensity of fluorescence in milk. Key: (a) FEU photomultiplier; (b) Vk, end switch; (c) IRN, source of regulated voltage.

In the case of individual milk specimens even with dilution with water [1, 4] a satisfactory convergence is generally observed in the results of the methods (within limits of 0.2%). This speaks

against any significant variability in the ratio of weight of tryptophan to weight of protein. Individual significant divergences (up to 0.4%) in the light of what has been said above may be related to differences in the degree of aggregation.

Thus, even with consideration of the possible insignificant deviation of constancy in the ratio of weight of tryptophan to the weight of protein the luminescence method is capable of ensuring an accuracy at which the absolute error will not exceed 0.1% protein. Here it is obvious that the most accurate results will be obtained on aggregate samples of milk.

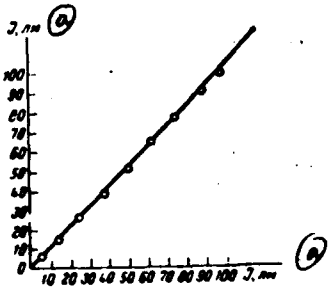

Fig. 5. Dependence of intensity of fluorescence of milk (dilution 1:10 with a layer thickness of 1 mm) and a standard (uranyl glass ZhS-9, 4 mm) on the intensity of excitation of light (width of diaphragm different).
Key: (a) I, lm.

In the process of developing an increase in the accuracy of the luminescence express method for the determination of protein in milk the authors naturally encountered the necessity for the maximum increase in the accuracy of the very process of measurement. The accuracy of the measurement process, generally speaking, should be sufficiently high in order to ensure the safe absolute error in industrial practice in the determination of protein content in milk (not exceeding 0.1%). Relative error in the measurement process should be less than 3%.

In connection with the fact that the sensitivity of photoresistors is insufficiently stable and changes depending on temperature and brightening, we were forced to avoid using them even though they were used in the initial investigations [1-3]. The compact FEU-25 photomultiplier turned out to be more satisfactory in operation. The photomultiplier has a glass cylinder and thanks to its high integral sensitivity and sufficient width for the band of protein fluorescence it turned out to be a completely suitable detector. For ensuring stable power supply to the photomultiplier we used a 6 V storage battery, the voltage of which was raised to 600 V with the help of a semiconductor converter. In order to stabalize the source of light (BUV-15 bactericidal uriol lamp) it was also fed from the storage battery. The arrangement of the appropriate voltage converter is depicted in Fig. 4. Due to stabilization of the voltage for feeding the lamp and the photomultiplier it was possible

to ensure the accuracy of measurements of the same dilution of milk up to 0.05-0.08% of absolute protein.

As a standard we used a luminescent screen made of ZhS-9 uranyl glass. The light fell on it after passing through a gaseous chlor--bromine filter and an UFS-1 light filter. With a change in the intensity of exciting light the changes in the intensity of the luminescent screen and the milk were directly proportional (Fig. 5). After establishing the intensity of exciting light according to a standard, one and the same samples of milk yielded similar readings for the course of several days (Table 4).

It is necessary to note that all the measurements were made in transmitted light, under conditions when parasitic (direct and diffused) light was approximately equal based on the intensity of the light of luminescence. Carrying out measurements at an angle of 90° to exciting light, as this is proposed by Koops and Wijnand, may further increase the accuracy of measurements. Further investigations are also necessary on the selection of the optimum conditions for measuring the intensity of luminescence of milk, but apparently without a doubt already in present form the method and the device can bring a definite benefit to industry, and the speeded up production of fluorescence protein meters by Soviet industry is a present day problem.

Conclusions

1. Factors which reduce the accuracy of the luminescence method of determination of protein in milk were analyzed.

2. Based on spectra of fluorescence, spectra of excitation and polarization of fluorescence of proteins in milk, it was shown that nonactive absorption of exciting light and the light of luminescence during excitation in the region of 250-280 nm should not lead to errors.

3. It was established that the majority of errors in analysis may be caused by differences in quantum yield of fluorescence of tryptophan in milk proteins developing as a result of differences in the dimensions or nature of packing of protein in granules. These differences are eliminated by means of dilution of milk in 8 M urea, leading to disaggregation of the protein and a reduction in the quantum yield of fluorescence.

4. Results were presented of a parallel determination of protein content in 21 aggregate samples of milk based on the Kjeldahl method (sample No 1), intensity of luminescence in water (sample No 2), and in 8 M urea (sample No 3). These showed that the absolute average divergence between samples No 3 and No 1 were less (0.07) than between samples No 2 and No 1 (0.14). The correlation coefficient between samples No 1 and No 3 comprised 0.9691, while for samples No 1 and No 2 it equaled 0.9475.

5. Several methods are proposed for increasing the accuracy of measuring the intensity of fluorescence of milk proteins taking into consideration the data obtained by Koops and Wijnand.

References

1. Konev, S. V., Kozunin, I. I., Zhivotnovodstvo, 5, 43, 1959.
2. Konev, S. V., Kozunin, I. I., Priroda, 11, 106, 1959.
3. Konev, S. V., Kozunin, I. I., In the collection Luminescence Analysis, Minsk, 1960.
4. Koops, J., Wijnand, Netherlande Milk and Dairy J. 15, 353, 1961.
5. Konev, S. V., Katibnikov, M. A., Pribory i Tekh, Eksperim., 4, 166, 1961.
6. Teal, F. W. J.,Biochem. J., 76, 81, 1961.
7. Vladimirov, Yu. A., Litvin, F. F., Biofizika, 5, 127, 1960.
8. Weber, G., Biochem. J., 75, 137, 1960.
9. Dyachenko, P. F., Investigation of Proteins in Milk, Tr. VNIMI, 19, Moscow, 1959.
10. Zaykovskiy, A. Ya., Chemistry and Physics of Milk and Dairy Products, Moscow, 1950.
11. Leviton, A., Haller, H., J. Phys. chem., 51, 460, 1947.
12. Vladimirov, Yu. A., Biofizika, 2, 116, 1957.
13. Vladimirov, Yu. A., Izv. AN SSSR, ser fiz., 23, 86, 1959.
14. Vladimirov, Yu. A., Burshteyn, E. A., Biofizika, 5, 385, 1960.
15. Konev, S. V., Katibnikov, M. A., Biofizika, 6, 638, 1961.
16. Katibnikov, M. A., Konev, S. V., Biofizika, 7, 150, 1961.
17. Brumberg, Ye. M., Meysel, M. N., Barskiy, I. Ya., Dokl. AN SSSR, 141, 723, 1961.
18. Konev, S. V., Certain Peculiarities of Photochemical Transformations in Biological Systems, Thesis, MGU, 1957.
19. Haugaard, J., Dam, M. G., Milchwissenschaft, 16, 452, 1961.
20. Raadsveld, C. W., Proc. 15th Int. Dairy Congr. olond, 3, 5, 1938, 1959.
21. Steiner, P. F., Edelhoch, H., Nature, 192, 873, 1961.