A C T A U N I V E R S I T A T I S L O D Z I E N S I S
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EFFECT OF ATP ON THE STABILITY OF ISOLATED APO-α AND APO-β CHAINS OF BOVINE HEMOGLOBIN*

Globin preparations were stored in the presence of ATP (molar ratio of ATP:Hb-tetramer equal to 2:1) in 8 M solution of deionized urea with the addition of 2-mercaptoethanol, at the temperature of +4°C. Under the same conditions control preparations, i.e. globin preparations without ATP were incubated. Separation of α and β globin chains was performed according to the method of Clegg on CM-32 cellulose columns and the content of α and β chains was determined after 24, 48, 72 and 96 h incubation. Purity of the obtained α and β globin chains was checked by isoelectrofocusing in polyacrylamide gel with 8 M urea and ampholine in the pH range of 3.5-10.0. Amino acid composition of α and β chains was determined by automatic amino acid analysis. It was found that the content of β chain in the control preparations decreased from 30 mg down to about 15 mg during 48-96 h storage. The content of β chains decreased down to about 21 mg in preparations stored with the addition ATP. The obtained results point to a stabilizing effect of ATP on the β chains of bovine globin. The content of α chains showed a slight decrease both in the control preparations and in the preparations stored in the presence of ATP.

Hemoproteins belong to proteins of low radiosensitivity. S z w e d a-L e w a n d o w s k a et al. [19] demonstrated e.g. that irradiation of 5% water solutions of human hemoglobin with

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doses of several Mrads of γ-radiation from a ^{60}Co source does not lead to a loss of the biological activity of this protein, i.e. the ability of reversible binding of oxygen. There exist an opinion that heme groups exert a protective action on polypeptide chains but this subject is not free from controversies.

The present studies on the effect of ionizing radiation on bovine hemoglobin are aimed at elucitation if the heme exerts a radioprotective effect on the primary structure of this protein. In the first stage of these investigations (W d z i ę c z a k, D u d a [22, 23]) it was found that irradiation of 1% water solutions of hemoglobin in the range of 1-5 Mrad results in a decrease of the content of -SH groups, histidine, tryptophan, tyrosine and proline. The next stage of these investigations was aimed at revealing of changes in the primary structure induced by irradiation of water solution of globin. This reqired obtaining of possibly stable globin preparation, and isolation of α and β globin chains.

W a t e r m a n and Y o n e t a n i [21] observed a considerable precipitation of apo-β chains of human hemoglobin during overnight dialysis and found that the yield of apo-β chains titrable with heme equaled to 2-10% while amounting to about 20% or more in the case of apo-α chains.

It has been previously reported (L e y k o, J ó ź w i a k [11]; L e y k o, W i e r z b i c k i [13]) that electrophoresis in starch gels containing urea enables revealing of a considerable augmentation of stability of apo-α and apo-β chains of human Hb by addition of ATP (1 µmole ATP per 0.2 µmole Hb-tetramer, and 3 µmole ATP per 0.2 µmole Hb-tetramer). This effect was especially striking in the case of apo-β chains. The control apo-β chain yielded a broad, heterogenous band in electrophoresis separation due to denaturation while a sharp, distinct, band was seen in separations of preparations stored in the presence of ATP (Fig. 1). Inhibition of denaturation changes of the apo-β chain was observed already at the ATP : Hb-tetramer molar ratio of 2 : 1.

The direct purpose of this study consisted in an examination of stability of apo-α and apo-β chains of bovine globin stored in the presence of ATP and their isolation according to the method of C l e g g et al. [1-3], applying ATP : Hb-tetramer molar ratio of 2 : 1.

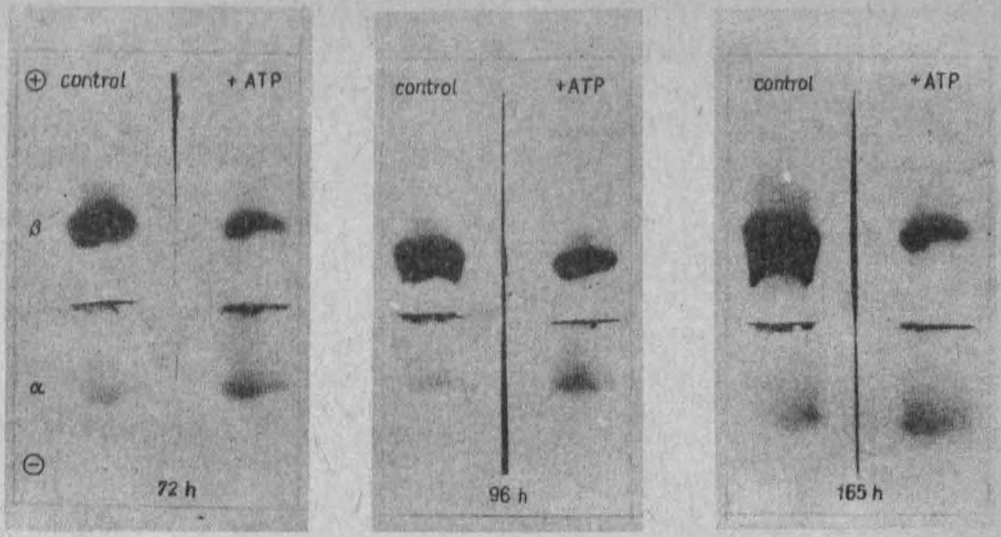

Fig. 1. Electrophoresis of apo-α and apo-β chains of human globin on urea starch gel, pH 8, 300 V, 22 h. Staining with amido black. Storage time 72, 96, 165 h [13]

Elektroforeza apo-α i apo-β łańcuchów globiny ludzkiej na mocznikowym żelu skrobiowym, ph 8, 300 V, 22 godz., barwienie czernią amidową, czas przechowywania 72, 96, 165 godz., wg [13]

Электрофорез апо-α и апо-β цепей человеческого глобина в крахмальном геле с мочевиной, pH 8, 300 V, 22 ч. Час хранения 72, 96, 165 часов, по [13]

MATERIAL AND METHODS

P r e p a r a t i o n o f h e m o g l o b i n. Hemoglobin from blood of Lowland Black and White Cattle was used for the investigations. This cattle breed is characterized by predominance of the type A of bovine hemoglobin, of wellknown amino acid sequence.

Bovine blood was taken in sodium citrate. The plasma was spun off. The obtained erythrocyte mass was washed 4 times with two volumes of cold 0.9% NaCl and the hemolysate was obtained by the method of D r a b k i n [5]. The hemolysate was dialysed (Visking 18/32 dialysis bag) against distilled water at the temperature of 4°C. Hemoglobin concentration in the hemolysate was determined spectrophotometrically, at the wavelength of λ = = 540 nm, in the cyanmet form, in a Spektromom 202 apparatus.

Preparation of globin. Globin was obtained from the hemolysate by precipitation with chilled acidified acetone [14]. 2 ml of 3% hemoglobin solution (60 mg Hb) was pipetted into 80 ml of acidified (3 ml 2 N HCl per 1 l) acetone, chilled down to -20°C. After centrifugation, the globin precipitate was washed 3 times with chilled acidified acetone for complete heme removal.

Preparation of α and β globin chains. α and β globin chains were isolated by the method of Clegg et al. [1, 2], adjusted for human globin. This method will be described in detail for the sake of the importance of successive steps for the interpretation of results. According to Clegg et al. [2, 3], globin precipitated with acidified acetone was dissolved in 2 ml of the starting buffer, containing 8 M urea, 0.05 M 2-mercaptoethanol and 0.005 M Na_2HPO_4. The buffer was adjusted to pH 6.7 with ortophosphoric acid. The globin was dialysed at room temperature against 50 volumes of the same buffer. The buffer was changed 3 times and the dialysis was performed for a total 2.5 h. The globin solution was applied to a carboxymethylcellulose column and following washing of the column(Ø 0.8 cm × 10 cm) with the starting buffer, separated into α and β chains in a linear gradient of Na^+ concentration. This gradient was obtained by appropriate mixing of 100 ml of the starting buffer with 100 ml of buffer containing 8 M urea, 0.05 M 2-mercaptoethanol and 0.03 M Na_2HPO_4 adjusted to pH 6.7 by ortophosphoric acid. In 1968, Clegg et al. [3] demonstrated that, after slight modification, this method can serve for separation of either hemoglobin or globin, yielding in each case globin α and β chains free from heme and non-heme proteins. Removal of heme is accomplished by washing of carboxymethylcellulose column with adsorbed globin with the starting buffer for 30-60 min. Under these conditions, mainly monomers of globin α and β chains are obtained.

In this study, the following conditions were applied: Two parallel globin samples (each prepared from 60 mg Hb) were evaporated to dryness at a room temperature and dissolved in 2 ml of the starting buffer (pH 6.8). Then one sample was added with 0.2 ml of 10 mM ATP solution· ($Na_2ATP \cdot 3H_2O$, from Sigma, purity

of 99%), what corresponded to 2 μM ATP per 1 μM hemoglobin tetramer. Both samples were stored at the temperature of +4°C, for 24, 48, 72 and 96 h, respectively, and then dialysed for 2 h against 50 volumes of deionized 8 M urea, pH 6.2. It results from the studies of G o n d k o and L e y k o [7] that considerable part of ATP remains bound to globin chains.

So prepared samples were separated into α and β chains on CM-32 cellulose (cationite, from Whatman, microgranulated) by the method of C l e g g et al. in a slight modification, indispensable for a good separation of α and β chains of bovine globin. 3 ml fractions were collected. First 10 fractions were eluted with deionized 8 M urea, pH 6.2, and the next 10 fractions with the starting buffer containing 0.0005 M Na_2HPO_4 in 8 M urea adjusted to pH 6.8 with ortophosphoric acid. The starting buffer applied in these expermients contained 10-fold lower Na_2HPO_4 concentration than the buffer used by C l e g g et al. [1-3] for separation of human globin chains. The next fractions (from 21 up to 110) were eluted with a linear gradient of Na^+ concentrations, obtained by appropriate mixing of 140 ml of the starting buffer with 140 ml of a buffer containing 0.03 M Na_2HPO_4 in 8 M urea, adjusted to pH 6.8 with ortophosphoric acid.

I s o e l e c r t i c f o c u s i n g Isoelectric focusing was applied in order to check the purity of α and β chains of bovine globin, separated on CM-32 cellulose. The following gel composition was maintained: 5% acrylamide, 0.2% bis-acrylamide (N,N'-methylene-bis-acrylamide), 12.5% sucrose, 8 M urea, 0.1% TEMED (N, N, N', N'-tetramethylethylenediamine), approximately 0.05% $(NH_4)_2S_2O_8$, 2% ampholine of the pH range 3.5-10.0 [4, 6].

After completed polymerisation (2 h, +4°C) the gel-containing tubes were mounted in an electrophoresis apparatus (LABOR-MIM, Hungary, type OE 107). 50 μl of a globin solution in 8 M urea (20 μg of protein) added with 25% sucrose was applied to the gel surface. The electrode vessels were filled with 0.2% H_2SO_4 and 0.4% ethyleneamine solutions. Electrophoresis was run until complete current breakdown. The initial current of 0.5 mA per tube. The gels were stained according to the method of V e s t e r b e r g [20] with the following staining solution: 150 ml ethanol, 352 ml distilled water, 60 g TCA (trichloroacetic acid),

18 g sulphosalicylic acid and 0.5 g Coomassie Brilliant Blue R 250. The staining was carried out at +60°C for 30 min. The background was de-stained with the following solution: 250 ml ethanol, 650 ml distilled water, 80 ml acetic acid.

Automatic amino acid analysis. α and β globin chains were subjected to acid hydrolysis in approximately 6 N HCl. Hydrolysis was run for 24 h, at the temperature of 110°C in sealed glass ampoules. The obtained hydrolysates were centrifuged (about 23 000 g, 20 min) and the sediment was evaporated to dryness over P_2O_5 and KOH (in substantia) in a vacuum desiccator. The evaporation procedure was repeated 3-4 times, the dry hydrolysate being always dissolved in thrice distilled water, until attaining pH of about 3. The automatic amino acid analysis was performed according to the method of Spackman, Stein and Moore [18], using a JOEL-JLC-6AH, Japan amino acid analyser, equipped with an integrator.

The adsorbed amino acids were eluted with 0.2 N sodium citrate, pH 3.25, 0.2 N sodium citrate, pH 4.25 and 0.35 N sodium citrate, pH 5.28. Identification and quantitative estimation of amino acids was based on comparisons of chromatograms with a chromatogram of a mixture of standard amino acids (concentrations of 0.1 μM/ml) and of integrator data.

RESULTS AND DISCUSSION

Isolation and characterization of α and β chains of bovine globin. Pattern of chromatographic separation of α and β chains of bovine globin, performed according to the method of Clegg et al. is presented in Fig. 2.

A small peak X precedes the β chain peak, and the α chain peak is preceded by an Y peak. For comparison, separation patterns of δ and β peaks and of α chains of Hb $Lepore_{Augusta}$ and HbA, obtained by Labie, Schroeder and Huisman [9] according to the method of Clegg et al. are given in Fig. 3.

According to these authors, X and Y peaks have slightly different compositions from those of appropriate main fractions but these differences to not seem to concern sulphur amino acids.

C l e g g et al. [3] performed separations of α and β chains for a mixture of HbA, HbE and HbF, using both hemoglobin and respective globins. In both cases they observed small peaks prior to peaks of γ and β^E chains, present in variable amounts, and yielding peptide maps, apparently identical with those obtained for α and β^E chains.

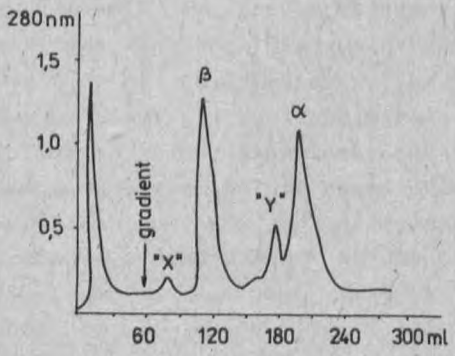

Fig. 2. The separation of the α and β chains of cattle globin on CM-32 cellulose according to the procedure of Clegg et al.

Rozdział α i β łańcuchów globiny wołowej na CM-32 celulozie metodą Clegga i wsp.

Разделение α и β цепей глобина быка на колонках с CM-32 целлюлозой методом Клегга и сотр.

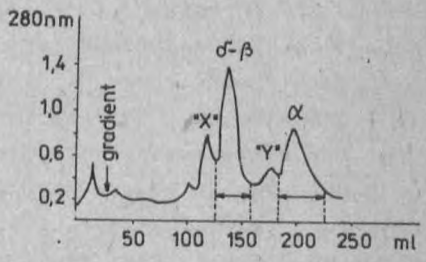

Fig. 3. The separation of the δ-β and α chains of hemoglobin Lepore$_{Augusta}$ on CM-cellulose according to the procedure of Clegg et al. [9]

Rozdział δ-β i α łańcuchów hemoglobiny Lepore$_{Augusta}$ na CM celulozie metodą Clegga i wsp., wg [9]

Разделение δ-β и α цепей гемоглобина Lepore$_{Augusta}$ на колонках с CM целлюлозой методом Клегга и сотр. [9]

According to H a n l o n et al. [8], one may assume that only $\alpha_2\beta_2$ tetramers and αβ dimers of bovine globin are present in the pH range of 4.4-7.1. Studies of these authors performed by the ultracentrifugation method (0.12%-0.17% solutions in phosphate and acetate buffers) revealed that at pH 7, the dimer-tetramer association constant ($2\alpha\beta \rightleftharpoons[K_2] \alpha_2\beta_2$) amounts to about $4 \cdot 10^6$ 1/mole for bovine globin, i.e. is higher than that found for human hemoglobin ($K_2 = 6 \cdot 10^5$ 1/mol at pH 7 by an order of magnitude).

The monomer-dimer association constant ($\alpha + \beta \rightleftharpoons[K_1] \alpha\beta$) in relatively high at pH 4.6 ($K_1 = 5 \cdot 10^5$) so the monomer frac-

tion is very small in acidic solutions (about 0.02%). This concentration is sufficient, however, for the exchange of free α and β chains under hybridization conditions leading to formation of α_2 and α_2. Nevertheless, the authors conclude that the relatively high affinity of α chains of bovine Hb for β chains of this Hb can not be the causative of the lack of capacity of this hemoglobin for hybridization.

It may be assumed that $\alpha_2\beta_2$ tetramers and αβ dimers occur under the conditions applied in this study and that the tetramers are more frequent than in analogous hemolysates obtained from human blood. Some further dissociation into α and β chains can take place during dropping of the hemolysate into acidified acetone. Basing on the analogy with human Hb one may expect that mainly α and β monomers are present in the solution obtained by dissolution of the precipitated globin in 8 M urea solution (pH 6.8). However, occurrence of some amounts of αβ, α_2 and β_2 and of $\alpha_2\beta_2$ tetramers seems also possible due to high association constants. It is considered [8] that the dimer-tetramer association involves proton release. It seems that under the conditions of separation on CM-cellulose according to the method of C l e g g et al., when applying linear gradient of Na^+ concentrations, the peak *X* might contain some $\alpha_2\beta_2$ tetramers and β_2 and some amounts of α_2 and αβ dimer might be present in the peak *Y*.

Preliminary studies of the *X* and *Y* peaks of bovine globin by automatic amino acid analysis revealed considerable differences in the amino acid composition with respect the composition of free α and β chains. As the main purpose of this study consisted in preparation of pure and native α and β globin chains, the additional peaks were not studied further, but only main peak fractions, corresponding to α and β globin chains were used for further investigations.

Purity of the obtained preparations of globin α and β chains was examined by isoelectrofocusing in polyacrylamide gel [4, 6] with ampholine of pH range 3.5-10.0 and 8 M urea, and by automatic amino acid analysis.

Polyacrylamide gel electrophoresis of the globin α and β chains, separated according to C l e g g et al. revealed only fractions corresponding to α and β chains. These results in-

dicate the lack of contamination of the globin α and β chains obtained after CM-32 cellulose separation by other proteins (Fig. 4).

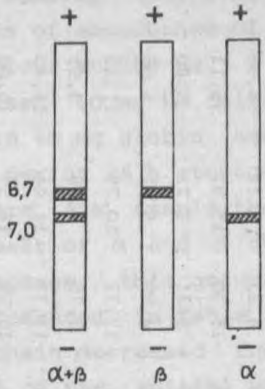

Fig. 4. Electrofocusing - scheme of the separation of α and β chains of cattle globin

Ogniskowanie izoelektryczne - schemat rozdziału α i β łańcuchów globiny wołowej

Изоэлектрическое фокусирование - схема разделения α и β цепей глобина быка

Both isolated globin α and β chains and their mixture were subjected to electrofocusing, what enabled determination of approximate pH values at which respective peptide chains attained pI. The pI values for α and β chains were equal to about 7.0 and 6.7 respectively.

Table 1 shows the found amino acid composition of globin α and β chains, expressed as so called "molar ratio" calculated by comparison of chromatograms of the examined samples with a chromatogram of a standard amino acid mixture.

The obtained results are compatible with the data of S c h r o e d e r et al. [15, 16] and with the results of own studies performed in the Istituto Superiore di Sanitá in Rome (in preparation). They confirm the fact that hemoglobin of Lowland Black-and White cows corresponds to Hb A type and point to a high purity of the obtained α and β chains. Homogeneity of the isolated chains was also by isoelectrofocusing.

Table 1

Amino acid composition of α and β chains of cattle globin

Skład aminokwasowy α i β łańcuchów globiny wołowej

Аминокислотный состав α и β цепей глобина быка

Amino acid	α chain				β chain					
	X	X	$\bar{X}$	N	X	X	X	X	$\bar{X}$	N
Lys	11.47	10.72	11.09	11	12.82	13.37	12.93	11.89	12.75	13
His	10.03	10.55	10.29	10	5.91	5.79	5.70	5.65	5.76	6
Arg	2.77	3.18	2.97	3	4.28	3.70	4.23	4.11	4.08	4
Asp = Asn	11.26	10.85	11.05	11	16.44	16.40	15.70	15.91	16.11	16
Thr	7.38	8.25	7.82	8	6.06	5.58	5.31	6.23	5.80	6
Ser	12.37	12.94	12.65	13	4.82	4.86	4.89	5.67	5.06	5
Glu = Gln	6.10	6.13	6.12	6	10.66	11.14	12.32	10.79	11.23	11
Pro	5.78	5.97	5.87	6	3.61	4.17	3.75	4.25	3.95	4
Gly	9.20	9.14	9.17	9	11.46	11.35	11.37	11.44	11.40	11
Ala	20.12	20.14	20.12	20	15.55	16.04	16.19	16.53	16.08	16
Val	12.09	12.16	12.12	12	17.87	17.68	17.68	17.88	17.78	18
Met	0.84	1.05	0.95	1	3.02	2.50	-	-	2.76	3
Leu	20.14	19.96	20.05	20	17.38	17.50	16.85	17.33	17.26	17
Tyr	3.06	2.79	2.93	3	2.05	1.84	-	1.82	1.90	2
Phe	7.42	7.60	7.54	7	10.49	9.51	10.67	9.53	10.05	10
Cys	-	-	-	0	-	0.99	0.90	-	0.95	1
Ile	-	-	-	0	-	-	-	-	-	0
Trp	-	-	-	1	-	-	-	-	-	2
Total			140.74	141					142.92	145

X - experimental data, $\bar{X}$ - mean values, N - nearest integer.

Effect of ATP on the content of α and β chains of bovine globin. The content of α and β chains in samples stored in the presence of ATP and in control samples (not supplemented with ATP) was calculated on the basis of absorbance of fractions obtained from chromatographic separations at 280 nm.

Each sample contained 60 mg Hb initially. It was assumed that the samples contain 60 mg globin and that the content of globin did not change during 24 h storage. Taking into account that globin β chains are less stable than α chains and that the areas under the peaks of α and β chains are practically equal following 24 h storage, this assumption seems justified. The obtained results are presented in Table 2.

The content of β chain decreased from 30 mg (24 h) down to about 15 mg (about 50% of the initial value) after 48-96 h storage in control samples and down to about 21 mg (about 70% of the initial value in the presence of ATP). The difference between both cases is statistically significant ($p < 0.01$) and indicate a stabilizing effect of ATP on the β globin chains.

The content of α chains did not exhibit significant changes in both control samples and in samples containing ATP during the incubation period applied (changes in the area under the peak of α chain were contained within the limits of the experimental error).

The stabilizing effect of ATP on globin β chains was found in cases of both bovine and human globin. The levels of ATP and DPG in human erythrocytes is considerably higher than in bovine red cells. Respective concentrations, expressed in μM/100 ml erythrocytes, amount to:

Human erythrocytes		Bovine erythrocytes
	(μM/100 ml erythrocytes)	
Adult	Adult	Calf	Adult
According to: [10]	[12]*	[17]**	[17]*
ATP-about 100	26.38 ±2.44	35.32 ±2.12	16.2 ±2.0
DPG-about 380	70.32 ±3.63	-	-

* 12 months old, ** 1 week old.

Table 2

Content of α and β chains in storage sample with ATP and control sample without ATP (in mg)

Zawartość α i β łańcuchów w próbie przechowywanej z ATP i kontrolnej bez ATP (w mg)

Содержание α и β цепей в пробах с АТФ и контрольных пробах без АТФ (в мг)

Storage time h	Control*			+ATP*		
	α + β	β	α	α + β	β	α
24	60	30.33	29.67	60	34.31	25.69
		s= 1.955	s= 1.896		s= 3.831	s= 5.830
48	45.56	12.93	32.63	48.61	19.57	29.04
		s= 1.106	s= 4.464		s= 1.896	s= 5.484
72	38.72	13.67	25.05	46.63	19.61	27.02
		s= 4.996	s= 6.560		s= 5.711	s= 8.730
96	47.77	18.96	28.81	56.48	23.48	32.26
		s= 2.688	s= 3.871		s= 1.854	s= 3.946

* mean values from 5 samples. The content of individual chains was calculated assuming that the total area under the peaks of α and β chains (100%) in samples stored for 24 h corresponds to 60 mg globin.

s - standard deviation.

One may suggest therefore that DPG or ATP can not affect the biological function of hemoglobin in bovine erythrocytes in the same manner as in human erythrocytes.

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WPŁYW ATP NA TRWAŁOŚĆ IZOLOWANYCH APO-α I APO-β ŁAŃCUCHÓW HEMOGLOBINY WOŁOWEJ

Preparaty globiny przechowywano w obecności ATP, stosując stężenie ATP: tetramer Hb = 2 : 1 w 8 M roztworze mocznika dejonizowanego z dodatkiem β-merkaptoetanolu, w temperaturze +4°C. W tych samych warunkach przechowywano próby kontrolne, tj. preparaty globiny bez ATP. Rozdziały α i β globiny wykonano metodą Clegga na kolumnach z CM-32 celulozą, oznaczając zawartość α i β łańcuchów globiny po 24, 48, 72 i 96 godzinach przechowywania. Czystość otrzymanych α i β łańcuchów globiny sprawdzano metodą ogniskowania izoelektrycznego w żelu poliakryloamidowym z 8 M mocznikiem i amfoliną o pH 3,5-10,0. Skład aminokwasowy α i β globiny oznaczano metodą automatycznej analizy aminokwasów. Stwierdzono, że w próbach kontrolnych zawartość łańcucha β zmniejszała się z 30 mg do ok. 15 mg w okresie 48-96 godzin przechowywania. W próbach przechowywanych z dodatkiem ATP zawartość łańcucha β zmniejszała się do ok. 21 mg. Uzyskane wyniki wskazują na na stabilizujący wpływ ATP na łańcuchy β. Zawartość łańcucha α zmieniała się nieznacznie zarówno w próbach kontrolnych jak i przechowywanych w obecności ATP.

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ВЛИЯНИЕ АТФ НА ПРОЧНОСТЬ ИЗОЛИРОВАННЫХ АПО-α И АПО-β ЦЕПЕЙ ГЕМОГЛОБИНА БЫЧЬЕЙ КРОВИ

Растворы глобина хранились в присутствии АТФ при концентрации АТФ: тетрамер Hb = 2 : 1 в деионизированной, 8 М мочевине, с добавлением β-меркаптоэтанола, т = +4°C. При таких же условиях

хранились контрольные пробы, т.е. растворы глобина без АТФ. Разделение глобинов α и β проводили методом Клегга на колонках с СМ-32 целлюлозой, определяя содержание цепей α и β после 24, 48, 72 и 96 часов хранения. Чистоту полученных цепей α и β проверяли при помощи метода изоэлектрического фокусирования на полиакриламидном геле с 8 М мочевиной и амфолином pH 3,5-10,0. Аминокислотный состав глобинов α и β определялся методом автоматического анализа аминокислот. Выявлено уменьшение содержания β-цепей в контрольных пробах от 30 мг до ок. 15 мг в течение 48-96 часов хранения. В пробах, хранившихся в присутствии АТФ, содержание β-цепей уменьшалось до ок. 21 мг. Результаты исследований свидетельствуют об стабилизирующем влиянии АТФ на β-цепи гемоглобина. Содержание α-цепей гемоглобина изменялось незначительно так в контрольных пробах, как в пробах, хранившихся в присутствии АТФ.