Synthesis, properties, and antianemic activity of new metal complexes of sodium pectinate with iron and calcium*

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A new water-soluble complex based on sodium pectinate with iron and calcium (P-NaFeCa) was synthesized. The regularities of the complexation of sodium pectinate (with a 35% content of COONa groups) with biogenic metals Ca and Fe were studied. The content of macro- and microelements was determined by atomic emission spectrometry; the product was identified by IR spectroscopy. Using atomic force microscopy, it was found that the average particle size of P-NaFeCa is ~170 nm. The efficiency of P-NaFeCa (per os) was studied for the first time in animals with hemolytic anemia modeled by administration of phenylhydrazine. It was experimentally shown that *in vivo* P-NaCaFe at a dose of 60 mg kg^{-1} containing 50% of the recommended therapeutic dose of iron helps to recover the number of erythrocytes and mean concentration hemoglobin in erythrocyte, which indicates the efficiency of P-NaCaFe as an agent restoring the blood parameters after exposure to hemolytic poisons.

Key words: citrus pectin, sodium pectinate, iron, calcium, complexation, IR spectroscopy, atomic force microscopy, antianemic activity, hemolytic phenylhydrazine anemia.

Pectin polysaccharides are polyuronide biopolymers present in almost all higher plants, sea grasses, and some algae.[1,2] They possess a wide range of physiological activity: immunomodulating,[3—5] antiinflammatory,[6,7] antioxidant,[8,9] as well as exhibit antidiabetic,[10] antitumor,[11] and prebiotic[12] effect. Pectins are registered as a natural food additive (E-440).[13]

Chemical modification of pectin polysaccharides makes it possible to obtain compounds with new physicochemical and physiological properties.[14] The Grindsted™ pectin XSS 100B was modified to iron polygalacturonates proposed for treatment of anemia.[15] The complexation of sodium polygalacturonate with cobalt and nickel led to the metal complexes exhibiting antimicrobial activity.[16] Antianemic activity is manifested by both polymetallic complexes based on sodium polygalacturonates simultaneously containing three microelements involved in the blood formation process, namely, iron, copper, and cobalt,[17] and metal complexes with only d-metal iron or s- and d-metals calcium and iron.[18] In the works of Japanese authors,[19,20] it was shown that a pronounced increase in the hemoglobin concentration in laboratory animals was caused by the use of iron pectate compared to the control group of rats administered with inorganic salts of divalent iron in equivalent doses.

Results and Discussion

The studies on the complexation of pectin polysaccharides with metal ions[16—18,21] are of undoubted interest. The present work is devoted to the synthesis of iron and calcium complexes based on sodium pectinate* and study of their physicochemical properties and antianemic activity. We propose the following approach to the synthesis of iron and calcium complexes based on sodium pectinate (P-NaCaFe) (Scheme 1): the first stage includes the synthesis of sodium pectinates with a 35% degree of salt formation at the free carboxy groups (the starting citrus pectin has a 65% degree of esterification). The resulting sodium pectinate (P-Na) serves as the starting ligand in the synthesis of metal complexes by the ligand exchange reaction of Na^+ ions with Ca^{2+} and Fe^{2+} cations, the target product (P-NaCaFe) is precipitated with ethanol, then the coagulated complexes are separated by centrifugation and lyophilized.

* Dedicated to Academician of the Russian Academy of Sciences A. I. Konovalov on the occasion of his 85th birthday.

* Pectate and pectinate is fully and partially deesterified salt of polygalacturonic acid, respectively.

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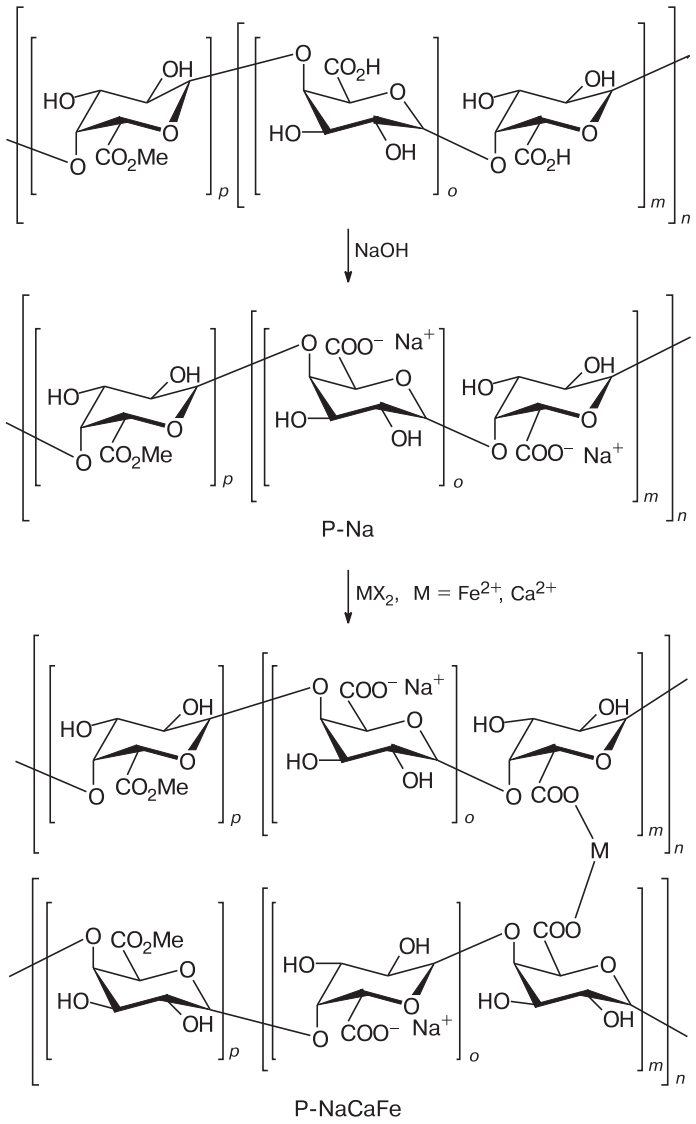
Scheme 1



The main difference is that in the previously developed approach to the synthesis of metal complexes of sodium polygalacturonates with iron and calcium (PG-NaCaFe), the first stage included the treatment of citrus pectin with an alkali under the controlled pH values at the titrimetric transition from a weakly acidic to the weakly basic region, which resulted in sodium pectate (polygalacturonate) with a 100% degree of salt formation. It should be emphasized that the conditions for obtaining iron and calcium complexes based on sodium pectinate are milder and less costly, since the complete deesterification of pectins to form sodium pectate with a 100% degree of salt formation is carried out at 50—60 °C for 2 h, while the synthesis of sodium pectinates (a 35% degree of salt formation) is possible at room temperature.

During the synthesis of the target product P-NaCaFe, we used IR spectroscopy to monitor the state of carboxy groups in the region of stretching vibrations of the COO^- group (1500—1800 cm^{-1}).[22] The ratio of the absorption intensities ν(C=O) may vary depending on what form predominates in the structure of pectin polysaccharides (ester, acid, or ionic). The IR spectrum of sodium pectinate (35% of COONa, Fig. 1, curve *2*) contains absorption bands of esterified carboxy groups characteristic of the starting citrus pectin (see Fig. 1, curve *1*). The IR spectrum of the iron and calcium complex with sodium pectinate (P-NaCaFe) is also shown in Fig. 1 (curve *3*). In the IR spectrum of sodium pectate (polygalacturonate) (PG-Na), an absorption band is observed in the region of stretching vibrations of the ionic form ν(COO^-) at 1610 cm^{-1}, while the absorption band of stretching vibrations of the carboxy or ester group ν(C=O) at 1745—1750 cm^{-1} is absent (see Fig. 1, curve *4*). The IR spectrum of sodium pectate (PG-Na) does not exhibit the absorption band δ(CH_3) at

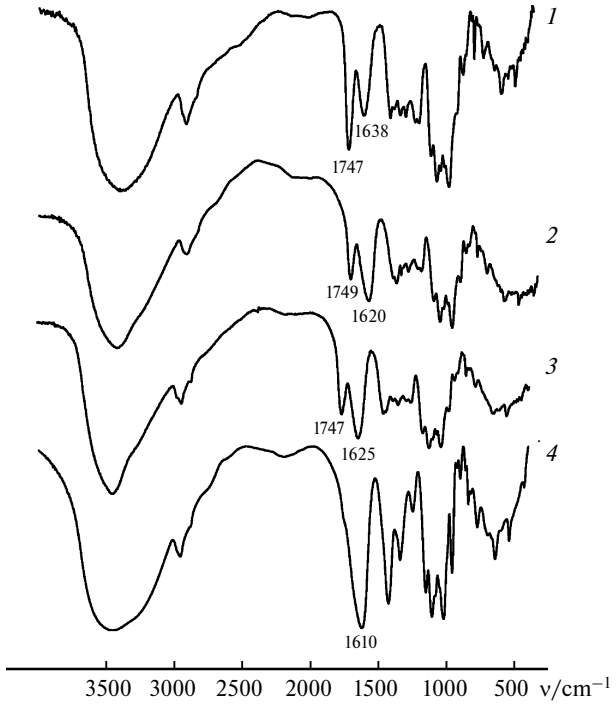


Fig. 1. IR spectra of citrus pectin (*1*), Na pectinate (*2*), NaCaFe pectinate (*3*), sodium pectate (100% COONa) (*4*).

1442 cm^{-1}, either, which is associated with the polysaccharide demethylation. This vibrational frequency of the C—H bond is characteristic of precisely methyl polygalacturonates (see Fig. 1, curve *4*).

The synthesized iron and calcium complexes with sodium pectinate are light brown amorphous powders, which form solutions in water at 50—60 °C with a concentration of up to 2%. The kinematic viscosity of 0.5% solutions of pectin metal complexes was determined by capillary viscometry and it was shown that the viscosity of pectinates P-NaCaFe is higher than that of pectates PG-NaCaFe (Table 1).

The content of metals in the iron and calcium complexes with sodium pectinate was determined by atomic emission spectrometry with inductively coupled plasma. In the target products (P-NaCaFe), the sodium content is 1.0 wt.%, while in the previously[21] synthesized iron and calcium complexes with sodium polygalacturonate (PG-NaCaFe) it is 4.6 wt.%, i.e., a significant decrease in sodium ions is observed with the same content of calcium and iron[21] (see Table 1). According to the World Health Organization, reducing sodium intake has a great potential to reduce the prevalence of cardiovascular diseases.[23,24] It is important for people prone to edema, liver and kidney disease. It should also be emphasized that the proposed version of the synthesis allows the preservation of the pectin native structures, since the degree of methoxylation remains at 65%.

The characteristics of pectin, iron and calcium complexes with sodium pectate and sodium polygalacturonate are presented in Table 1. It is noted that all the complexes are optically active.

The morphology of species obtained by precipitation of metal pectinates and pectates from solutions (the concentration of solutions was 10—4 wt.%) was studied by atomic force microscopy (AFM) on a MultiMode V scanning probe microscope (Veeco, USA) (Fig. 2). The samples were prepared by applying a drop of the solution on a mica substrate with subsequent deposition of these species onto it. Figure 2 shows that P-Na is characterized by the formation of nonuniform species with two maxima in the size distribution histogram: ~100 and ~1200 nm, while the PG-Na, P-NaCaFe, and PG-NaCaFe species are characterized by a unimodal distribution with average sizes of 110, 170, and 55 nm, respectively.

Currently, within the framework of the "Preclinical studies of innovative medicines" event of the Federal Target Program "Development of the pharmaceutical and medical industry of the Russian Federation for the period up to 2020 and beyond", preclinical studies of the drug based on Na-, Fe-, Ca-polygalacturonate for treatment of anemia are conducted, which confirm its efficiency. We assume that the newly synthesized iron and calcium complex with sodium pectinate is also promising, which makes it necessary to evaluate its pharmacotoxicological characteristics in laboratory animals.

The study of the toxicological properties of the synthesized compound P-NaCaFe was conducted in accordance with the Guidelines for Conducting Preclinical Studies of Drugs[25] and in compliance with the requirements of the European Convention on the Protection of Vertebrate Animals used for experimental and other scientific purposes. The trials showed that in the acute toxicity test with a single intragastric administration of the compound in a dose of 20,000 mg kg^{-1} to white laboratory

Table 1. Characteristics of pectin metal complexes

Sample	Na	Fe	Ca	C	H	$[\alpha]_D^{20}$	Kinematic viscosity /mm^2 s^{-1} (20 °C)
	wt.%						
P-NaCaFe	1.0±0.05	1.1±0.05	2.3±0.11	31.32±0.17	4.17±0.13	+191.5 (*c* 0.5, H_2O)	2.68 (*c* 0.5, H_2O)
PG-NaCaFe	4.6±0.23	1.1±0.05	2.3±0.11	27.66±0.23	3.52±0.28	+186.0 (*c* 0.25, H_2O)	1.93 (*c* 0.5, H_2O)
Pectin	—	—	—	36.51±0.15	6.48±0.01	+255.7 (*c* 0.5, H_2O)	2.36 (*c* 0.5, H_2O)

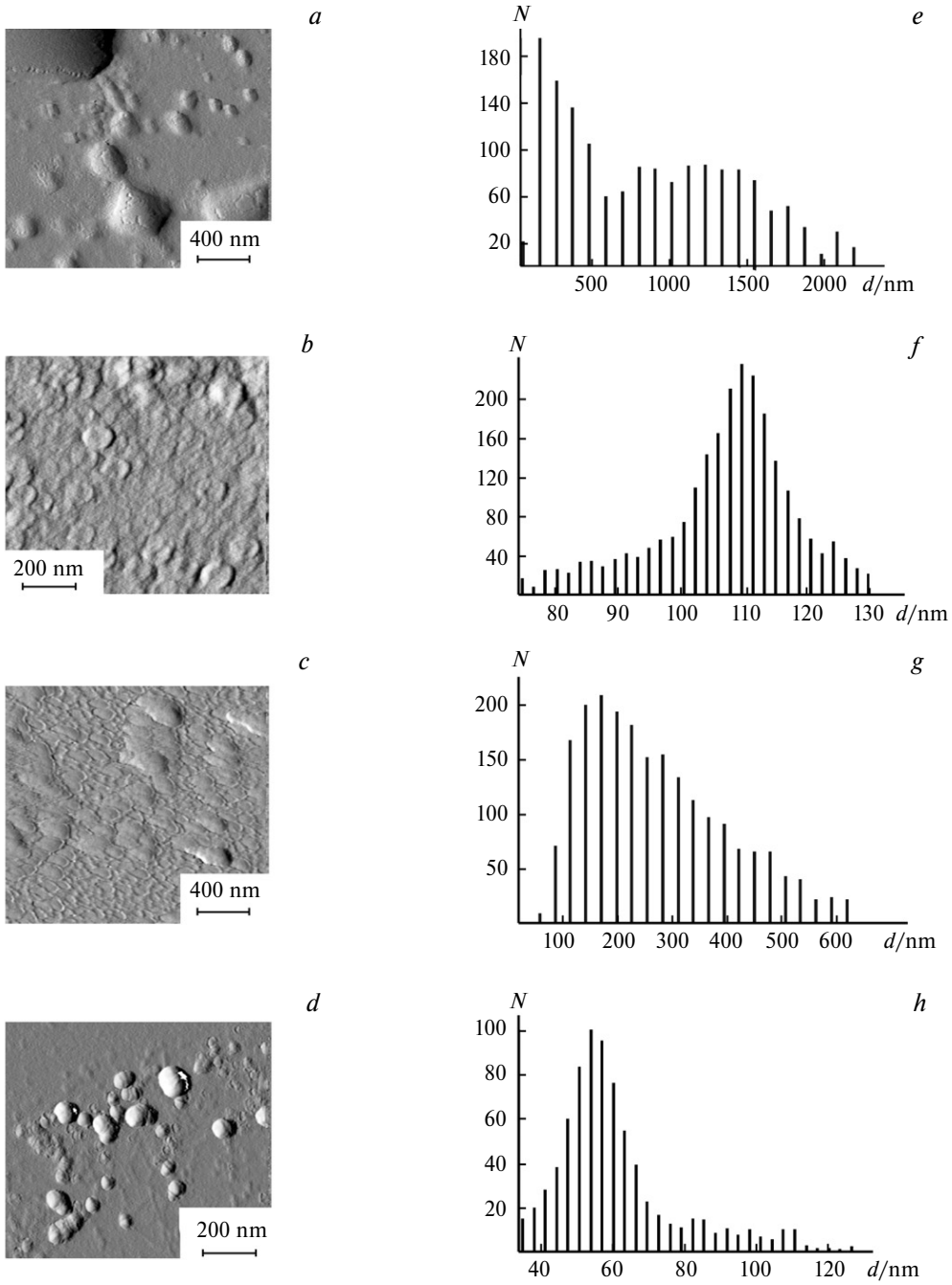


Fig. 2. The AFM image of sample species (*a*—*d*) and their size distribution (*e*—*h*): P-Na (*a*, *e*); PG-Na (*b*, *f*); P-NaCaFe (*c*, *g*); PG-NaCaFe (*d, h*).

rats, no animal death was observed, which rates the synthesized compound as the IV class low toxic substance (LD50 higher than 5000 mg kg^{-1}, USSR State Standard Specifications 12.1.007-76).

Study of the efficiency of P-NaCaFe in the phenylhydrazine anemia. The antianemic activity of the P-NaCaFe complex was studied on a rat hemolytic phenylhydrazine anemia model. It was shown that the three-fold administration of phenylhydrazine did not lead to a statistically significant change in the level of hemoglobin, which is characteristic of the toxic phenylhydrazine anemia. The dynamics of hemoglobin change in the control group after the introduction of phenylhydrazine was characterized by its gradual increase by the 3rd week of the experiment by 28.7% relative to the initial values (the absolute differences were statistically significant at $p < 0.01$, the deviation from

Table 2. The results of the study of hematological parameters in laboratory animals with hemolytic phenylhydrazine anemia

Day of test	Concentration of Hb/g L^{-1}			Number of red blood cells (RBC)/$\times 10^{12}$ L^{-1}			Mean content hemoglobin in a separate erythrocyte (MCH)/pg		
	1st group	2nd group	3rd group	1st group	2nd group	3rd group	1st group	2nd group	3rd group
Initially	143.8±6.9	143.4±5.8	147.7±5.2	7.8±0.2	7.7±0.2	**8.2±0.1**	18.8±0.5	19.0±0.4	18.8±0.5 (*)
4th (after administration of PH)	153.9±15.5	141.1±8.9	148.0±7.4	8.4±0.4	8.4±0.2	8.1±0.1	17.2±1.1	17.1±0.9	17.2±0.3
7th	159.6±1.2	158.4±1.9	**168.0±1.9** * (*)	3.5±0.6 (***)	2.9±0.3 (***)	3.2±0.3 (***)	42.6±3.7 ***	49.5±2.9 ***	48.3±3.9 ***
14th	168.0±1.9 (*)	163.8±5.0 (*)	**173.4±2.0** (**)	4.8±0.8 (**)	4.3±0.3 (***)	4.8±0.3 (***)	37.1±8.0 (*)	37.9±3.3 (***)	35.3±1.8 (***)
21st	185.0±4.4 (**)	183.1±6.4 (**)	173.1±3.4 (**)	5.6±0.5 (**)	6.4±0.3 (**)	5.6±0.5 (**)	31.0±3.2 (**)	25.5±0.7 (***)	31.7±2.6 (**)
35th	146.9±3.1	146.9±2.7	**154.1±3.5**	7.4±0.1 (**)	7.8±0.2	**8.2±0.1** ***	24.9±0.3 (***)	23.5±0.8 (**)	21.2±0.3 *** (***)

Note. The asterisks without brackets indicate differences with the control group, in brackets the differences with the initial values.
* Statistically significant at $p < 0.05$.
** The same at $p < 0.01$.
*** The same at $p < 0.001$. Positive effect of test compounds is highlighted in bold.

the initial values was significant at $p < 0.05$ in the 2nd week). Then a decrease in hemoglobin was observed by the 4th and 5th week to the level of the initial values (Table 2).

It was experimentally shown that the most pronounced increase in hemoglobin level relative to the initial values at the end of the experiment (5th week of observation) was in the groups which received the P-NaCaFe complex. The differences in hemoglobin level relative to the initial values were: 1st group (control) 102.2%, 2nd group (administered with the drug Totem) 102.4%, 3rd group (administered with P-NaCaFe) 104.3%.

Administration of P-NaCaFe resulted in the most pronounced increase in hemoglobin in the 1st and 2nd weeks. The dynamics of hemoglobin change in the group received the drug Totem did not differ from the control: the hemoglobin level in the test groups, like in the control group, decreased to the initial values, slightly exceeding them.

When exposed to hemolytically active toxic compounds, the most important is to study the changes in the number of erythrocytes, since they undergo destruction under the action of hemolytics. It was shown that on the 7th day of the experiment, a more than two time decrease in the number of erythrocytes in rats was observed; the differences with the initial values in all groups were statistically significant at $p < 0.001$ (see Table 2). At the same time, the parameters of an individual erythrocyte underwent significant changes: the mean content hemoglobin (MCH) more than doubled; the differences with the initial values are statistically significant at $p < 0.001$. The number of erythrocytes and MCH in the control group recovered to the standard values only by the 5th week of the experiment, however, the number of erythrocytes remained lower the initial level by 5.1% and MCH above it by 32.4%. In the group received the drug Totem, the number of erythrocytes recovered in the 5th week of the experiment, but MCH remained 23.7% above the initial level.

With the administration of P-NaCaFe, the number of erythrocytes on the 5th week of the experiment exceeded the corresponding level of the control group by 10.8%, but did not differ from the initial level. In this group, the MHC in the 5th week of the experiment was lower than the corresponding control values by 14.9%, but remained higher than the initial values by 12.8% (see Table 2). The results showed that the administration of the P-NaCaFe complex in the case of hemolytic phenylhydrazine anemia contributes to a more efficient recovery of the studied hematological parameters in comparison with both the control group and the group administered with the drug Totem in a dose containing an equimolar amount of iron.

In conclusion, a new water-soluble complex based on sodium pectinate with iron and calcium (P-NaCaFe) was synthesized. Some aspects of the complexation of sodium pectinate (with a COONa group content of 35%) with biogenic metals Ca and Fe were studied.

The quantitative content of macro- and microelements was determined by atomic emission spectrometry, the product was identified by IR spectroscopy, an average particle size for the P-NaFeCa sample was determined by AFM and found to be 170 nm. For the first time, the efficiency of P-NaCaFe in oral administration in a dose of 60 mg kg^{-1} containing 50% of iron from the recommended therapeutic dose was studied in animals with phenyl-

hydrazine hemolytic anemia model. It has been established that the administration of P-NaCaFe contributes to a more efficient recovery of blood parameters, namely, the number of erythrocytes and the mean hemoglobin content in an individual erythrocyte, compared to the control and the group of animals administered with Totem with an equimolar iron content. The results indicate the efficiency of P-NaCaFe as an agent restoring blood parameters in the toxic effects of hemolytic poisons.

Experimental

IR spectra were recorded on a Tensor 27 IR spectrometer (Bruker, Germany) in KBr pellets. The metal content was determined on an iCAP 6300 DUO atomic emission spectrometer with an inductively coupled plasma (Thermo Scientific, USA). The pH values were determined on an I-160MI laboratory ionomer. Optical rotation was measured on a Perkin-Elmer 341 polarimeter (concentration c in g (100 mL)$^{-1}$, wavelength 589 nm, temperature 20 °C). The elemental composition was determined on a EuroEA3028-HT-OM high-temperature CHNS-O analyzer (Italy). AFM images of the surface of the P-Na, PG-Na, P-NaCaFe, PG-NaCaFe samples, as well as the particle size distribution, were obtained on a MultiMode V scanning probe microscope (Veeco, USA).

Iron and calcium complexes with sodium pectinate were synthesized on the basis of the Classic C-401 citrus pectin (Herbstreith & Fox, Germany). The metal salts used for the synthesis, iron sulfate heptahydrate and granulated anhydrous calcium chloride, met the requirements of the USSR State Standard Specifications 4148-78 and 4460-79, respectively; NaOH (USSR State Standard Specification 4328-77, amendment 1, 2) and other reagents were of analytical purity grade.

Synthesis of sodium pectinate (P-Na). Distilled water (1.5 L) was placed into a 2-L flask with constant magnetic stirring, followed by a portionwise addition of citrus pectin (40 g) and heating to 50—60 °C until complete dissolution of pectin. A solution of NaOH (1.8 g) in distilled water (100 mL) was then added in small portions to the pectin solution at controlled pH values. Next, the volume of the reaction mixture was brought to two liters and the synthesis of sodium pectinate was performed at room temperature for 1 h with continuous stirring. IR (KBr), ν/cm^{-1}: 1748, 1616 (COO$^-$).

Synthesis of sodium pectinate with iron and calcium (P-NaCaFe). Solutions of calcium chloride (0.44 g) and iron sulfate (0.36 g) in distilled water (200 mL of each) were successively added to a sodium pectinate solution (450 mL) with stirring. The mixture was kept at 50—60 °C for 15—20 min and cooled to room temperature. The complex was precipitated with a double volume of 95% ethanol. The precipitate was separated by centrifugation (Sigma, Germany), then freeze-dried (Alpha 1-2 LD: Martin Christ, Germany). The weight of the iron and calcium complex with sodium pectinate was 7.3 g. IR (KBr), ν/cm^{-1}: 1748, 1620 (COO$^-$).

Examination of antianemic activity of P-NaCaFe. The efficiency of iron and calcium complexes based on sodium pectinate with a COONa group content of 35% was investigated for the first time in animals with modeled phenylhydrazine hemolytic anemia in the vivarium of the A. E. Arbuzov Institute of Organic and Physical Chemistry, of the Kazan Scientific Center of the Russian Academy of Sciences. The studies were performed on outbred white rats. The animals according to the method of analogs were divided into three groups of 4—5 individuals in each. The average weight of animals in the groups was 295.0, 275.3, and 316.0 g in the 1st, 2nd, and 3rd group, respectively.

The animals initial blood parameters were examined before the experiment. Then, hemolytic anemia was induced by subcutaneous administration of phenylhydrazine at a dose of 30 mg kg^{-1} (3% solution at a rate of 0.1 mL per 100 g of weight) for three days, after which hematologic parameters were re-examined.

After that, the test drugs were injected daily for 35 days intragastrically through an atraumatic probe:

— 1st group (control) received water in the amount of 0.2 mL kg^{-1};

— 2nd group (with the comparison substance) received the antianemic drug Totem at a dose of 0.12 mL kg^{-1} (before the administration, the content of the ampoule was diluted by a factor of 8.3 and injected at the rate of 0.1 mL per 100 g of the body weight);

— 3rd group (with the test compound) received P-NaCaFe as a 3% solution at the rate of 0.2 mL per 100 g of the mass, the dose of 60 mg kg^{-1}.

The animals in groups received P-NaCaFe and Totem, the same amount of iron was administered, which calculated on the body weight was 0.6 mg kg^{-1} per day (based on 50% of the dose, which was 1.3 mg kg^{-1} recommended for the treatment of anemia for iron sulfate-based drugs).[15]

Hematological parameters were investigated on the 7th (three days after the introduction of phenylhydrazine), the 14th, 21st, and 35th days of the experiment. The following parameters were examined: hemoglobin (Hb) by the hemoglobin cyanide photometric method, the number of red blood cells (RBC) by counting in the Goryaev chamber, the mean content hemoglobin in a single red blood cell (MCH) using calculation by the formula

$$\mathrm{MCH(pg)} = \mathrm{Hb(g\ L^{-1})/Er(10^{12}\ L^{-1})}.$$

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