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Determination of the composition and functional activity of the conjugates of colloidal gold and antibodies

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This article describes a method for estimating the composition of conjugates of gold nanoparticles with antibodies and its binding capacity for the antigen. The conjugates are separated in first order from the unbound antibodies by centrifugation after synthesis, and then the concentration of unbound antibodies in the supernatant is analysed by an enzyme immunoassay (ELISA). Conclusions regarding the composition of these conjugates are made on the basis of the difference between the concentrations of the added and the unbound antibodies. This approach protects against the influence of nanoparticles on the label, and the high precision of the immunosorbent assay can reliably detect even small changes in the concentration of antibodies caused by the immobilisation. The amount of antigen binding to the obtained conjugate is registered, and thus the stored reactivity of immobilised antibodies is assessed in the same system. The developed method was applied to characterise colloidal gold conjugates with anti-species antibodies (sheep antibodies to human immunoglobulin). It is shown that in the course of the interaction between the immobilized sheep antibodies and free human immunoglobulins, not more that 12% of the binding sites of the sheep antibodies are able to bind the human immunoglobulins.

Keywords: antibodies, gold nanoparticles, conjugates, enzyme-linked immunosorbent assay

INTRODUCTION

To date, labelled antibodies are one of the most frequently used detection agents in bioanalytical systems [1-3]. Enzymes, dyed lattices and, in many cases, gold nanoparticles (GNPs) are used as labels in these systems, such as immunochromatography [4,5]. Visualisation using antibodies labelled with gold nanoparticles has undeniable advantages due to the unique optical properties of these nanoparticles and their stability, high surface area and ease of modification [6].

Although the obtaining of analytical reagents based on GNPs is described in a large number of papers, a number of important methodological questions are not

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yet solved, such as the ability of antibodies to bind to the surface of GNPs and the effect of the amount of antibodies in the conjugate on its binding ability.

To date, many techniques have been proposed for the experimental measurement of the number of molecules adsorbed on the surface of nanoparticles [7], including spectroscopic methods (absorption [8], emission [9], fluorescent [10] and others), mass spectrometry [11] light scattering techniques (Raman [12], dynamic light scattering [13] and others) and analytical separation techniques (chromatography [14], electrophoresis [15], analytical ultracentrifugation [16], flow field fractionation [17] and others).

Various methods of analysing nanodispersed conjugates have produced radically different results [10]. For example, some studies argued that proteins form a monolayer on the nanoparticle surface [18-20], whereas multilayer immobilisation has been observed in other studies [21,22]. Depending on how the interaction of protein and gold nanoparticles was determined, the values obtained by different methods may vary by five orders of magnitude for the same protein [9,20,23-25]. This can be explained by the insufficient accuracy of some methods of analysis, the influence of the assay conditions on the conjugate composition (a conjugate under stringent conditions may undergo partial destruction) and the influence of nanoparticles on adsorbed molecules and labels (e.g. colloidal gold may be a powerful modulator of fluorescence intensity [26,27]).

In contrast to the conjugates' composition, the stored reactivity of immobilised antibodies has been characterised to a much lesser degree. However, this issue is of great importance since it is known that nanoparticles can have a significant effect on the properties of the adsorbed proteins on their surface [22].

In this paper, we propose a simple technique based on an enzyme immunoassay (ELISA) to determine the total number of antibodies on the gold nanoparticle surface and the amount of antibodies that retained the ability to bind to an antigen. The developed method was applied to determine the composition and functional activity of the colloidal gold conjugate with anti-species antibodies, i.e. sheep antibodies, sIgG, to human immunoglobulin, hIgG.

The number of anti-species antibodies adsorbed on the surface of the gold nanoparticles was determined according to the following:

1. Incubation of the nanoparticles with sIgG.
2. Separation of free sIgG from the conjugates by centrifugation.
3. Determination of the concentration of free sIgG by ELISA.
4. Calculation of the composition of the conjugates on the basis of material balance.

The ability of the conjugates to bind to hIgG antibodies was determined by the following:

1. Incubation of conjugates GNPs-sIgG with hIgG antibodies.
2. Separation of the free hIgG from the conjugates by centrifugation.
3. Determination of the concentration of free hIgG by ELISA.
4. Calculation of the binding capacity of the conjugates GNPs-sIgG based on material balance.

EXPERIMENTAL

Reagents

Sheep immunoglobulin G to human immunoglobulin G (sIgG, anti-species antibodies), human immunoglobulin G (hIgG) (Imtek, Russia). Tween-20, sodium azide, 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma, USA). Chloroauric acid (Fluka, Germany). Bovine serum albumin (BSA) (MP Biomedicals, USA).

Buffer and support solutions

50 mM phosphate buffer (PBS), pH 7.4

To prepare 1 litre 5.85 g NaCl, 2.4 g of KOH and 6.8 g KH_2PO_4 were added to 1 litre of distilled water. The solution was stirred with a magnetic stirrer and kept at + 4°C.

50 mM phosphate buffer, pH 7.4, containing 0.05% Triton X-100 (PBST)

500 ul of detergent Triton X-100 was added to 1 litre of PBS and thoroughly mixed with a magnetic stirrer and kept at + 4°C.

0.1 M carbonate buffer, pH 9.0

First, 100 mL of a 0.1 M sodium carbonate were prepared, and 2.862 g of crystalline salt decahydrate was dissolved in 100 mL of bidistilled water. Next, a 900 mL 0.1 M sodium bicarbonate was prepared by dissolving of 8.4 g of salt in 900 mL of water. The resulting solutions were mixed in a ratio of 1:9, and the pH of the resulting solution was adjusted with concentrated hydrochloric acid.

Sodium citrate buffer (pH 6, 100 mM)

This buffer solution was prepared by dissolving 0.242 g of citric acid monohydrate and 2.60 g of sodium acetate dihydrate in 100 mL of bidistilled water. The resulting solution was stirred with a magnetic stirrer and kept at + 4°C.

TMB Substrate

The stock solution was prepared by dissolving 10 mg of TMB in 1 mL dimethyl sulfoxide (DMSO) while being stirred in a shaker. The resulting solution was stored at + 4°C and protected from light. For the substrate preparation, 100 mkL of TMB stock solution and 20 mkL of hydrogen peroxide (3% aqueous solution) were added to 10 mL of sodium citrate buffer (pH 6; 100 mmol) and mixed thoroughly.

10 mM Tris-HCl, pH 9.0

To 100 mL of 0.2 M tris (hydroxymethyl) aminomethane, 20 mL 0.1 M HCl were added, the volume was adjusted to 2 litres of bidistilled water and pH was adjusted with concentrated HCl.

The solution "ttBSA"

2.0 mL of 10% BSA solution, 0.2 g of sucrose, 1 mL of 2% sodium azide and 8.95 mL of water were added to 8.0 mL of 10 mM Tris-HCl pH 9.0.

Colloidal gold synthesis by the Frens method

For colloidal gold synthesis, 2.95 mL of 0.34% chloroauric acid was added to 97.5 mL of boiling deionised water, and the mixture was heated and stirred for 2 minutes. Then, 1.44 and 0.92 mL of 1% sodium citrate solution were added, boiled for 30 minutes and cooled to room temperature [28]. Colloidal gold was stored at + 4°C.

Determination of the dimensional characteristics of gold nanoparticles and the presence of aggregates in the prepared conjugates by electronic microscopy

Preparations of GNPs and conjugates were applied to copper hexagonal 200 mesh grids coated with formvar film. Microscopic analysis was performed using a «Jeol JEM – 100 CX / SEG» (Japan, 66,000 magnification) transmission electron microscope. The obtained micrographs were treated using Image Tool software (University of Texas Health Science Center at San Antonio, San Antonio, USA). The largest and smallest size, the average diameter and the ratio of the axes [29] were calculated for each particle.

Conjugation of colloidal gold nanoparticles with antibodies

0.2 M of K_2CO_3 solution was added to the GNP preparation by drops to reach a pH of 9.0. Sheep IgG antibodies against human IgG were dialyzed against 10 mM carbonate buffer pH 9.0 [30].

sIgG solution was added to the tubes with 1 mL GNPs. Added sIgG concentrations during conjugation were 5, 10, 30, 50 and 93 mkg/mL. Incubation was provided for 30 min with stirring at room temperature. After this, 25 mkL of 10% aqueous solution of BSA was added. The obtained mixture was incubated for 15 min with stirring at room temperature.

The resulting conjugates were centrifuged for 15 min at 10,000 g at +4°C, with decanting of the supernatant liquid. Then, the precipitate (40 mkL) was redissolved in 1 mL of ttBSA. Centrifugation, decantation and the addition of ttBSA were repeated two more times. All supernatants were saved.

ELISA of free unbound sIgG

100 mkL of hIgG at 1 mkg/mL in PBS was added to each microplate well and incubated for 2 h at 37°C. The plate was then washed four times with PBST. To obtain the calibration curve, a series of dilutions of sIgG or supernatants collected after centrifugation of sIgG-GNPs conjugates were added to the microplate wells and incubated for 45 min at 37°C. After washing the microplate with PBST, 100 mkL of peroxidase-labelled mouse anti-sIgG antibodies (dilution of the commercial product in PBST was 1:3000) was added to each well and incubated for 45 min at 37°C. After washing, the peroxidase activity of the resulting complexes was measured. For this purpose, 100 mkL of TMB substrate solution was added to each microplate well and incubated for 10 min at room temperature. The enzymatic reaction was terminated by adding 50 mkL of 1 M H_2SO_4 to each well. The optical density of the oxidation product was measured at 450 nm.

ELISA of hIgG not bound with the conjugate

100 mkL of rabbit anti-hIgG antibodies (1 mg/mL in PBS) was added to each microplate well and incubated for 2 h at 37°C. The plate was then washed four times with PBST. To obtain the calibration curve, a series of dilutions of hIgG in PBST was added to the wells and incubated for 1 h at 37°C.

The sIgG-GNPs conjugate (the concentration of antibodies during the synthesis was 30 mkg/mL) was concentrated 10-fold to obtain a particle concentration of 10 nM. 100 mkL of PBST was added to 50 mkL of the concentrated conjugate to prevent nonspecific adsorption. Then 150 mkL of human hIgG solutions was added to obtain final hIgG concentrations of 5, 10, 30 and 50 mkg/mL. The mixtures were incubated for 30 min at room temperature and then centrifuged for 20 min at 10.000 g and 4°C. Supernatants were collected and transferred to the microplate well with the immobilised rabbit anti-hIgG antibodies. The microplate was incubated for 1 h at

37°C and washed four times with PBST. The following incubation with peroxidase-labelled mouse anti-sIgG antibodies and the measurement of the peroxidase activity were carried out as described in the previous section.

RESULTS AND DISCUSSION

Preparation and characterisation of gold nanoparticles

Gold nanoparticles were prepared by reducing of gold (III) chloride hydrate ($HAuCl_4$) with sodium citrate [28]. The dimensions of the resulting particles were determined using transmission electron microscopy. The dimensional characteristics obtained after the analysis of the microphotographs (Figure 1) using Image Tool software are presented in Table 1. The average particle diameter was 20.7 nm (evaluated from 210 particles).

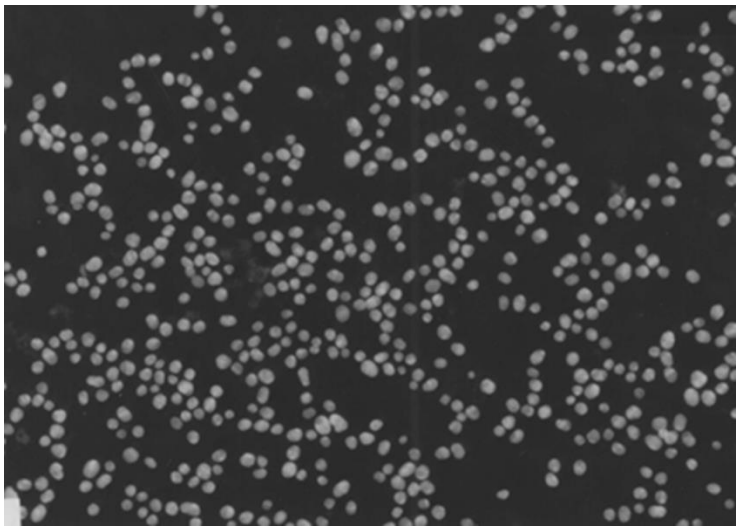

Figure 1. Microphotographs of gold nanoparticles with a diameter of 20 nm. Images were obtained using an enlargement of 66,000.

Table 1. Characteristics of gold nanoparticle size and homogeneity.

	Major axis length, nm	**Minor axis length, nm**	**Ratio of axles**
The average value	22.8	18.5	0.845
Standard deviation	4.1	2.9	0.095

Determination of the composition of the conjugates of antibodies with gold nanoparticles

To determine the amount of antibodies in the conjugates, a calibration curve for the ELISA of sIgG was obtained as described above (Figure 2). The linear dependence was observed up to 1.0 units of optical density (OD). The correlation coefficient was 0.99. The initial part of the calibration curve was linearised according to the following equation:

$$OD = 0.12 + 10.7 \times C, \quad (1)$$

where 0.12 is a background signal of peroxidase substrate.

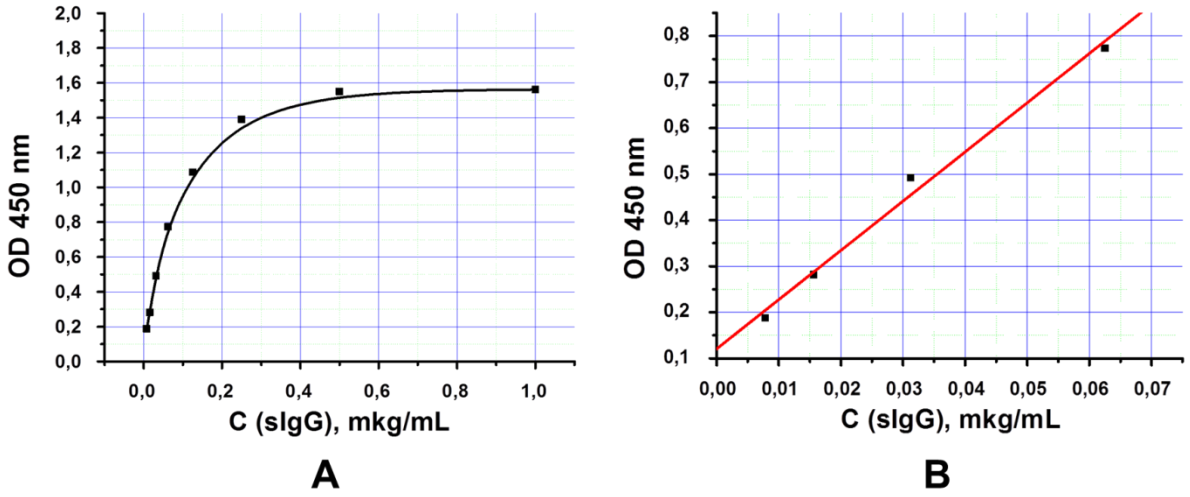

Figure 2. A. The dependence of the optical density at 450 nm on sIgG concentration in the sample. **B.** The linear part of this dependence.

To determine the concentration of free unbound sIgG in the reaction mixtures, ELISA of the supernatants collected after centrifugation of the conjugates was carried out. For each supernatant, eight serial (sequential) 2-fold dilutions were prepared. The linear parts of the obtained dependences of the optical density on sample dilutions were used to determine the sIgG concentration on the basis of Equation 1. The same experiments and calculations were performed for the supernatants obtained after re-centrifugation of the conjugates. All concentrations were calculated taking in the account the volume of the reaction mixture during conjugation of GNPs with antibodies. The concentration of sIgG in the conjugate was calculated by subtracting the calculated concentration of free sIgG from the initial sIgG concentration. The corresponding calculations for various sIgG-GNPs conjugates of different compositions are given in Table 2 and Fig. 3. The data demonstrate that varying the sIgG initial concentration from 5 to 93.5 mkg/mL results in 92–74% binding of sIgG. This parameter decreases with the increasing concentration. However, saturation is not observed in the specified concentration range, which is consistent with the data described by Bell et al., who did not observe a complete saturation of the surface of the GNP preparations, with a diameter in the range of 20 to 80 nm, even at IgG concentrations more than 1 mg/mL [13].

Table 2. Characteristics of antibody binding to gold nanoparticles at different antibody loading.

Initial sIgG concentration, mkg/mL	**sIgG concentration in the 1st supernatant, mkg/mL**	**sIgG concentration in the 2nd supernatant, mkg/mL**	**sIgG concentration calculated in the conjugate, mkg/mL**	**Conjugated antibodies, %**
5	0.23	0.15	4.62	92
10	0.70	0.23	9.07	91
30	4.47	0.54	24.98	83
50	9.56	1.30	39.14	78
93.5	23.21	1.05	69.23	74

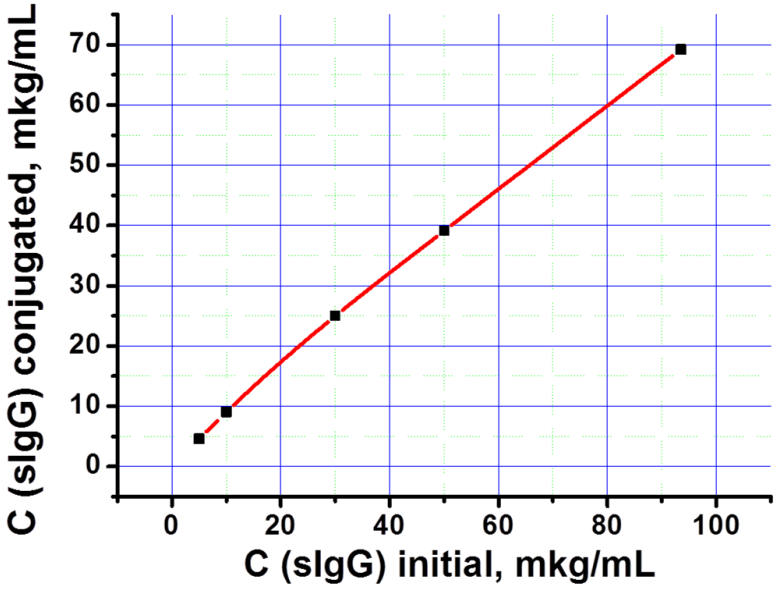

Figure 3. The dependence of sIgG concentration in the conjugate on the initial sIgG concentration.

Determination of the functional activity of conjugated antibodies

To determine the ability of the conjugated sIgG to bind an antigen, we incubated sIgG-GNPs conjugates (30 mkg/mL during conjugation) with hIgG. As it was shown in the previous section, after adding 30 mkg/mL of sIgG, only 25 mkg/mL of sIgG was bound with nanoparticles. In order to increase the amount of bound hIgG, the sIgG-GNPs conjugate was concentrated 10-fold. The mixing of the conjugate with the hIgG solution resulted in its 6-fold dilution (3-fold in PBST and 2-fold with a solution of hIgG). Therefore, the final amount of sIgG in the solution was 41.7 mkg/mL. Four hIgG concentrations were used in the experiment: 50, 30, 10 and 5 mkg/mL (final concentrations in the reaction solution). After incubation of the conjugates with hIgG solutions, the reaction mixture was centrifuged and the amount of hIgG in the supernatants was analysed by ELISA (four samples with different concentrations of hIgG). Fig. 4 shows the dependence of the optical density at 450 nm on hIgG concentration. The initial part of the calibration curve (at an optical density of less than 1.0) was linearised by the following equation:

$$OD = 0.02 + 13{,}4 \times C. \quad (2)$$

where 0.02 is a background signal. The correlation coefficient was 0.98.

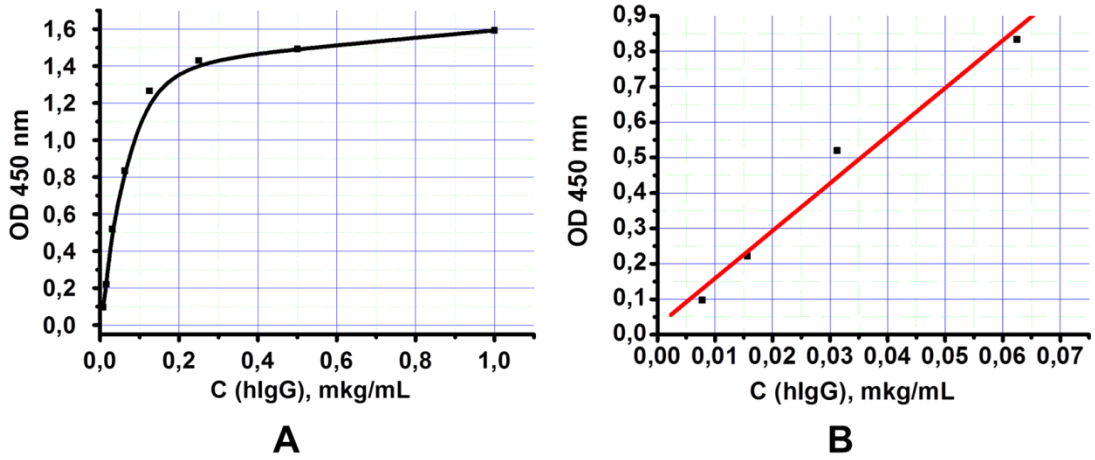

Figure 4. The dependence of the optical density at 450 nm on concentration of human IgG in the sample.

For all four supernatant samples, eight serial (sequential) 2-fold dilutions were prepared. Then hIgG concentration was determined by ELISA in each of them. Values selected from the linear region of the obtained calibration curves were used to evaluate hIgG concentrations according to Equation 2. Based on these data, the concentration of hIgG bound with a conjugate was calculated. The values obtained for different initial hIgG concentrations are shown in Fig. 5.

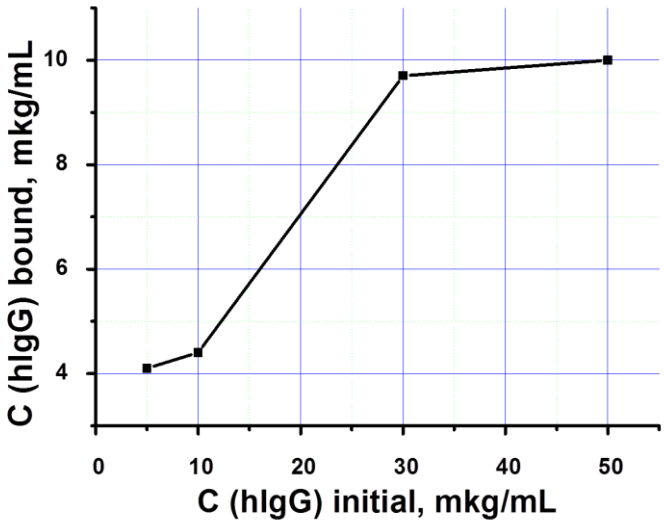

Figure 5. The dependence of the concentration of human IgG bound with the sIgG-GNPs conjugate on the initial human IgG concentration.

The maximum amount of hIgG bound with a conjugate was 10 mkg/mL. The concentration ratio of the bound hIgG and sIgG in the conjugate was 0.24. Moreover, since each antibody has two valencies, each sIgG molecule could potentially bind to two hIgG molecules. Therefore, only 12% of the potential valencies were occupied.

This can be explained by several reasons. The first is the undirected adsorption of antibodies on the gold surface, which results in loss of antibody ability to bind to an antigen. The second relates to the fact that human IgG is a bulk antigen that may obscure one or more antigen binding sites on the surface of the conjugate. It is also possible to form the multilayer structures of the adsorbed antibody molecules on

the surface of the conjugates, whereby the inner layers of the adsorbed antibody molecules become unavailable for the antigen. It should be noted that there is no consensus in the literature on whether IgG molecules form mono- or multilayers on the surface of GNPs. Various studies have demonstrated both multilayer [21] and monolayer [10] IgG immobilisation, which could be due to differences in the conditions of the synthesis of the conjugates.

CONCLUSION

IgG-GNPs conjugates are among the most commonly used labelled reagents in biochemical studies and analytical systems. However, issues regarding composition and binding capacity remain debatable due to the limitations of the analytical methods used. The proposed method for determining these parameters is simple and accurate, and it can be used to optimise the composition of the conjugates. The pre-separation of the conjugate from the sample solutions allows for the avoidance of the influence of nanoparticles on the label properties, and it increases the accuracy of the analysis. In this paper, this technique was applied to the conjugates of gold nanoparticles with secondary antibodies; however, this approach is universal and can be applied to other antibody–antigen pairs.

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