# A novel method, based on lithium sulfate precipitation for purification of chicken egg yolk immunoglobulin Y, applied to immunospecific antibodies against Sendai virus.

*G. Bizhanov[1], I. Jonauskiene & J. Hau[2]**

*[1]Laboratory of Biomodels, Institute of Immunology, 29 Moletu plentas, LT-2021 Vilnius, Lithuania;*

*[2]Department of Neuroscience, Division of Comparative Medicine, Uppsala University BMC, Box 572, 75 123 Uppsala, Sweden*

***Summary***

Egg-laying hens were immunized with Sendai virus (SV) that had been grown in chicken embryos. The titres of immunospecific SV antibodies varied from $\log_2 12$ to $\log_2 16$ during the 5-month immunization period and total immunoglobulin Y (IgY) concentrations varied from 1.6 to 4.0 mg per ml of egg yolk. Two IgY purification methods based on salt precipitation using lithium sulfate or sodium citrate were developed. These methods were compared with two other purification methods based on polyethylene glycol (PEG) precipitation and chloroform extraction, respectively in terms of yield, total protein content, IgY concentration and immunospecific anti Sendai IgY activity. The total protein and IgY contents when purified by chloroform were 1.4-2.8 times and 1.3-2.3 times higher, respectively than in corresponding preparations purified by the other methods. However, the proportion of nonsense proteins was approximately 10% higher in the IgY preparation purified by chloroform than in those purified by salt precipitation. The immunospecific IgY activity recorded in the preparations from the new salting out methods was lower compared with the PEG and chloroform purification methods. However, the purity analysis of IgY by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) demonstrated that IgY purified with lithium sulfate contained only two major components with molecular weights of 40 kDa and 66 kDa and one minor protein component of 45 kDa. By contrast, IgY extracted with ammonium sulfate, which is a classical method used to obtain purified IgY, contained two major protein of 40 kDa and 66 kDa and at least three less intense protein bands corresponding to proteins of molecular weights 31.4 kDa, 33.5 kDa and 45 kDa. The results indicate that the purification of IgY by lithium sulfate results in very pure IgY in high quantities (94% +/- 5% of total egg yolk protein).

***Introduction***

There is an increasing interest in the use of chicken egg yolk for polyclonal antibody production for practical and economical reasons *(Svendsen et al. 1994, Bollen & Hau 1996, Tini et al. 2002))* and chicken egg yolk antibodies (IgY) have been applied successfully for scientific *(Schade et al.1997),* diagnostic *(Di Lonardo et al. 2001)*, prophylactic *(Almeida et al. 1998)* and therapeutic purposes *(Lemamy et al. 1999)*. Because of the phylogenetic distance between birds and mammals, mammalian proteins are often more immunogenic in birds than in other mammals and antibody synthesis readily stimulated in hens *(Leslie & Clem 1969)*. In addition, because of the phylogenetical distance bird antibodies against a mammalian protein may often react with analogous proteins in other mammalian species *(Hau et al. 1980, 1981)*. An additional advantage of the chicken system for antibody production is that many of the viruses used to induce antibodies can be grown in fertile eggs in order to avoid anti-host

* *Correspondence:* Jann Hau
Telephone: + 46 18 471 46 22 · Fax: + 46 18 50 17 40
E-mail: jann.hau@bmc.uu.se

antibodies *(Schild & Dowdle 1975)*.

Egg yolk IgY has a molecular weight of 180 kDa, which is higher than that of mammalian IgG, lower isoelectric points and slightly different physico-chemical properties compared with mammalian IgG *(Bollen & Hau 1996)*.

From an animal welfare point of view chickens are an attractive alternative to mammals as antibody producers because large quantities of antibodies can be produced from the egg yolk making restraint and blood sampling obsolete techniques to the benefit of the animals used for this purpose *(Erb & Hau 1994, Svendsen et al. 1994, Schade et al. 1996, Hendriksen & Hau 2002)*. A further improvement of the conditions for chickens used in antibody production may well result from oral immunizations techniques, which are being developed *(Hoshi et al. 1999, Klipper et al. 2001, Hedlund & Hau 2001)*.

Hens can be immunized for production of polyclonal antibodies against a large variety of antigens as reviewed by Bollen (1997). The large amount of lipid in egg yolk *(Hatta et al.1988)*, however, renders some purification of IgY necessary for scientific use regardless of the assay in which they are to be used. Several methods were described in the 1950ies for purifying IgY based on the strategy of separation of proteins (levitins) from lipoproteins (lipovitellins) and the rest of the yolk lipids using extraction with organic solvents with rather low yields of antibody *(Jensenius et al. 1981)*. However, purification methods based on organic solvents like chloroform remain in use *(Ntakarutimana et al. 1992)*. Other methods are based on affinity chromatography *(Greene & Holt 1997)* or on dilution of the yolk followed by a freezing-thawing process after which the process consists of ion exchange chromatography and/or salt precipitations often combining a number of salts like e.g. polyethylene glycol (PEG) *(Jensenius et al. 1981, Polson et al. 1985)*, dextran sulfate *(Jensenius et al. 1981)*, dextran blue *(Bizhanov & Vyshniauskis 2000)*, sodium sulfate *(Jensenius et al. 1981)*, ammonium sulfate *(Jensenius et al. 1981, Svendsen et al. 1995)* caprylic acid *(Svendsen et al. 1995)* and sodium citrate *(Akita & Nakai 1993)*. More recently methods combining chloroform removal of lipids with ammonium sulfate precipitation techniques have been shown to result in a good yield of antibodies of high purity *(Hedlund & Hau 2001)*.

The main aims of the present study were i) to produce immunospecific IgY antibodies to Sendai virus and ii) to test the use of lithium sulfate in the purification process for IgY against three other existing methods: chloroform extraction, PEG-6000 precipitation and sodium citrate precipitation.

## *Materials and Methods*

### *Animals and husbandry*

Twelve 25-week-old outbred Brown Hisex hens were obtained from the breeding unit of Vaisa, the laboratory animal resources of the Institute of Immunology (Vilnius, Lithuania). Three female chinchilla rabbits 3-5 months old and 1.5-2.0 kg body weight were obtained from the same breeder.

The hens and rabbits were kept singly in 1 m x 1 m floor pens equipped with nest boxes in a standard animal room with a 17/7 h light/dark cycle. As bedding, chips of deciduous trees were used, after sterilization at 120º C, pressure 1.5 kg/cm$^2$ during 20 min. The bedding was changed twice weekly.

The temperature in the room was 20$^o$ C±2$^o$ C, with a relative humidity within the range of 55%-60% and the noise level was maintained below 50 dB.

The chicken and rabbit feed was based on granulated diet ("Biosynthesis" AB Vilnius, Lithuania). This consisted of dry matter (88%), crude protein (20%), fat (3%) and carbohydrate (4%). The feed was balanced for vitamins and micronutrients, and the moisture content did not exceed 12%. Water was provided *ad libitum*.

### *Preparation of viral antigen*

Sendai virus of the Fushimi strain was obtained from the Institute of Virology (Moscow, Russia) and grown for 72 h at 34$^o$C in the allantoic fluid of 9-day-old chicken embryos. The virus was harvested and cleaned by centrifugation at 2000 x g, 40$^o$C

for 20 min, then concentrated with 8% PEG-6000 (Fluka AG, Buchs, Switzerland). The mixture was stirred at 4°C overnight and centrifuged at 5000 x g, 10°C for 20 min. The pellet containing the virus was resuspended in tris-buffered saline (TBS) (10 mM Tris, 150 mM NaCl, pH 7.5) and dialyzed against this solution at 4°C for 24h. The virus was then inactivated by treatment with 0.05% (v/v) formaldehyde at 4°C for 24h. This viral sample was used to immunize the hens.

*Immunization of hens with Sendai virus*

The viral suspension (100 µg in 500 µl of TBS) was emulsified with an equal volume of complete Freund's adjuvant (Calbiochem, Corp., La Jolla CA, USA). Twelve laying hens were immunized by injection of 1 ml of this emulsion distributed into four sites of the pectoral muscle of each bird. A second immunization using incomplete Freund's incomplete adjuvant (Calbiochem) was performed after 27 days. Eggs were collected daily, beginning 5 days after the first injection, and stored at 4°C until analysis.

*Immunization of rabbits with chicken IgY*

Three rabbits were immunized intramuscularly in the thigh using a dose of 5 mg highly purified IgY in 1 ml TBS emulsified in an equal amount of Freund's complete adjuvant (Calbiochem). On day 14 the rabbits received a booster injection with a similar dose of antigen emulsified in incomplete Freund's adjuvant. Blood was collected 10 days after the final inoculation from the marginal ear vein and allowed to clot for about 1 h at room temperature. The clot was then loosened with a glass rod after which the blood sample was stored at 4°C overnight. The clot-free serum was transferred into a tube and centrifuged for 20 min at 1500 x g at 4° C.

*Haemagglutination inhibition assay*

Anti-Sendai virus antibody titres were measured by haemagglutination inhibition. Serial 2-fold dilutions in 50 µl of TBS of egg yolk before (control samples) and after immunization were prepared in duplicates in 96 well plates (U-shaped bottom, Nunc A/S, Roskilde, Denmark). Four haemagglutinin units of the Sendai virus in 50 µl were added to each well and the plates were incubated at room temperature for 1h; afterwards 50 µl of 1% chicken erythrocytes were added. The results were read after incubation for 2h at 4°C. The antibody titres were expressed as the reciprocal of the highest dilution of IgY at which more than 90% inhibition of haemagglutination was observed.

The titres were converted to a base-2 logarithmic scale. The geometric means (GM) were calculated from $GM=\Sigma\ antilog_2/n$, where the numerator is the sum of the antilogarithms of all titre values (in $log_2$) and the denominator is the number of samples *(Hoy 1992)*.

*Purification of IgY from egg yolk*

The yolks of 10 eggs were collected 7 weeks after the second immunization and egg yolks were separated from egg whites, washed with distilled water to remove as much albumen as possible and rolled on paper towels to remove adhering egg white. The yolks were pooled, mixed and a mixture of TBS and egg yolk (4:1, v/v) was prepared. From this mixture aliquots were processed according to the five different protocols.

1. Precipitation with PEG-6000 (Fluka) following the procedure described by Polson et al. (1985);
2. Chloroform extraction as decribed by Ntakarutimana et al. (1992);
3. By the water dilution method described by Akita and Nakai (1993), but replacing sodium sulfate by sodium citrate and
4. As method #3 but replacing sodium sulfate by lithium sulfate and
5. As method #3 but replacing sodium sulfate by ammonium sulfate.

In methods 3 - 5 the yolk was diluted 1:9 with distilled water, acidified with 100 mM HCl to reach pH 5.0 and incubated at 4°C overnight. After further centrifugation at 7000 x g, 10°C for 20 min, the precipitate containing IgY was resuspended in TBS, precipitated twice in either sodium citrate, lithium

sulfate or ammonium sulfate (34 % final concentration) and dialyzed against TBS. The procedure was repeated at least three times for each method, with essentially the same results.

*Preparation of standard IgY*

IgY was purified from egg yolk with PEG-6000 (*Polson et al*.1985) as described above and then dialyzed overnight using 5.0 mM phosphate buffer pH 6.5.

DEAE-52 (16 g) (Whatman, International Ltd, Maidstone, Kent, UK) was equilibrated using several washes in 5.0 mM phosphate buffer, pH 6.5, washes and packed by centrifugation at 1000 x g for 20 min at room temperature. The DEAE-52 was then mixed with the IgY solution containing 7.0 mg/ml of total protein and incubated for 1 h at room temperature. After centrifugation at 1000 g for 20 min at room temperature, the supernatant containing the IgY was dialyzed overnight against TBS at 4°C and used to immunize the rabbits and as standard IgY in the radial immunodiffusion assay. When this IgY preparation was analyzed for purity by SDS-PAGE (*Laemmli 1970*), it contained two protein components of 40 kDa and 66 kDa.

*Radial immunodiffusion assay*

Radial immunodiffusion (RID) *(Mancini et al. 1965)* was used to estimate the concentration of total IgY in egg yolk. Rabbit anti-chicken IgY antisera were used in the assay at a concentration of 1.6% (v/v) in 1% (w/v) agarose (type II, Sigma, St. Louis, MO, USA) and TBS. Dilutions (1:1 to 1:8) of purified chicken IgY (3.4 mg/ml) was used as standards to generate standard curves by plotting the zone annulus area (the area of zone minus the area of well) of the precipitation rings after 18 h diffusion in a moist chamber at room temperature. The IgY content (mg/ml) of test samples was calculated from the standard curves according to Schild et al.(1975). Precipitation rings were measured to the nearest 0.1 mm using a ruler (Bio-Rad, Lab., Richmond, CA, USA).

*Polyacrylamide gel electrophoresis*

Polyacrylamide gel electrophoresis (SDS-PAGE) was performed in homogenous slab gels (0.75 mm x 16 mm x 180 mm) of 4% acrylamide (Serva, Heidelberg, Germany), with 2.7% bisacrylamide (Serva) in the stacking gel, using a Protean II electrophoresis system (Bio-Rad) with the discontinuous buffer system of Laemmli (1970). The samples were dissolved in 1% SDS, 2% 2-mercaptoethanol, 0.625 mol/L Tris, pH 6.8, and heated at 100°C for 5 min. Electrophoresis was done at 15 mA for 5 h at 4°C. The gel was stained for protein with silver nitrate *(Oakley et al. 1980)*.

*Total protein estimation*

The protein content of the product obtained by the different methods was determined *(Lowry et al. 1951)* using a standard curve generated with bovine serum albumin (Sigma) at concentrations from 0 to 0.2 mg/ml.

*Statistical analysis*

The mean antibody titres of the chicken sera were compared using Student's *t*- test. All values were expressed as mean ± standard deviation and differences with p values $<0.05$ were considered significant.

***Results***

*IgY titres off egg yolk antibodies with specificity against Sendai virus.*

Specific Sendai virus antibodies were detected at week 1 post immunization (Figure 1). Following reimmunization, the level of specific antibodies continued to increase above $\log_2$ 12 throughout the 20 weeks period of observation. The titres were below $\log_2$ 3 in the controls.

*Concentration of total IgY in egg yolk.*

The IgY concentration in egg yolk increased steadily during the immunization period until week 8 where it reached 4 mg/ml. After week 8 the levels decreased gradually to reach a level of 2.5 mg/ml.

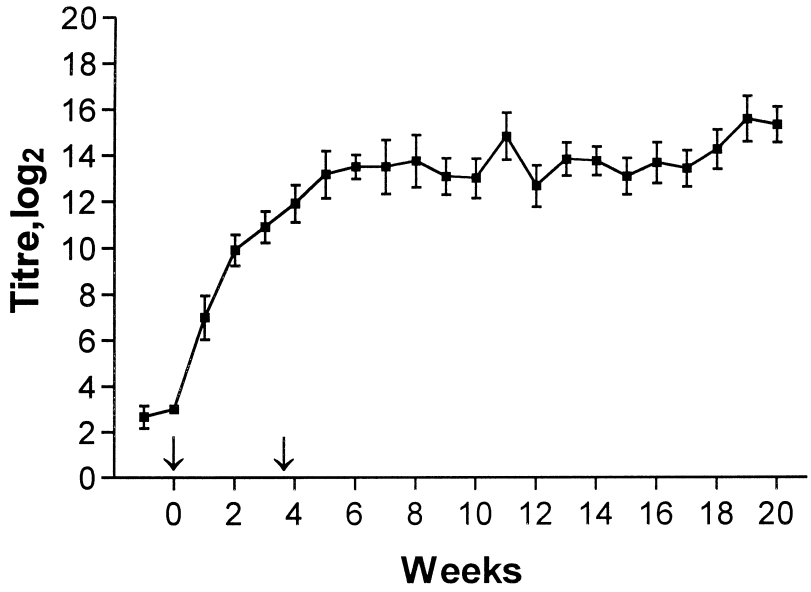


Figure 1. Level of Sendai virus antibodies in egg yolk purified by polyethylene glycol (PEG) 6000. Anti-Sendai antibody virus titres were measured by haemagglutination inhibition test. The arrows indicate the time of immunization.

*Quantitative characteristics of the IgY preparations purified by the four different methods.*

The IgY preparations purified by use of chloroform contained significantly more total protein as well as IgY than did those purified by the three other methods (Table 1). The proportion of IgY of the total protein isolated by chloroform was only about 80% as compared with more than 90% in the IgY preparations purified by salt precipitation.

The immunospecific anti Sendai virus activity was higher in the IgY preparations obtained by the methods based on PEG precipitation and chloroform extraction compared with the activity of the IgY preparations purified by the two other methods.

The concentrations of total protein and IgY purified by PEG precipitation were significantly lower than those in corresponding preparations purified by the three other methods.

*Qualitative comparison of IgY preparations purified by ammonium sulfate precipitation and by lithium sulfate precipitation.*

The purity of IgY purified with lithium sulfate was analyzed by SDS-PAGE and the gel demonstrated two major protein components with molecular weights of 40 kDa and 66 kDa, and one less intense band corresponding to a protein of 45 kDa. (Figure 3). IgY purified by ammonium sulfate contained two dominating proteins of 40 kDa and 66 kDa, and three bands of less intensity corresponding to proteins of molecular weights 31.4 kDa, 33.5 kDa and 45 kDa. (Figure 3).

***Discussion***

As early as one week after the initial immunization of laying hens with Sendai virus immunospecific antibodies were found present in high titre in the egg yolk. The titre increased steadily during the following five weeks, and remained at a fairly steady high

Table 1. The characteristics of immunoglobulin Y (IgY) purified from egg yolk by polyethylene glycol (PEG) 6000, chloroform and water dilution methods[a].

[a]Egg yolk collected from eggs 7 weeks after the second immunization. [b],[c] Results with different suffixes differ significantly from results in the same column.

| Method of purification | Total protein (mg/ml of egg yolk) | IgY (mg/ml of egg yolk) | Mean geometric anti-Sendai virus titre ($log_2$) | IgY/ protein (%) |
|---|---|---|---|---|
| PEG-6000 | $4.6^{b}$±0.4 | $4.4^{b}$±0.4 | $13.3^{b}$±0.1 | 95.6±3.6 |
| Chloroform | $13.2^{c}$±0.7 | $10.7^{c}$±0.6 | $11.7^{c}$±0.5 | $81.0^{b}$±1.0 |
| Lithium sulfate | 8.2±0.2 | 7.7±0.2 | 8.9±0.5 | 93.9±4.8 |
| Sodium citrate | 9.1±0.7 | 8.2±0.7 | 8.6±0.5 | 90.1±4.0 |

level throughout the 20 weeks observation period. This confirms our earlier observations *(Bizhanov & Vyshniauskis 2000)*, whereas lower titres have been reported in eggs of chickens immunized with Newcastle Disease Virus *(Piela et al. 1984)*. These differences may be due to immunogenicity differences between the viruses as well as to the use of different immunization schemes, different breeds of hens and different assays employed *(Losch et al. 1986; Bizhanov & Vyshniauskis 2000)*. Other reports

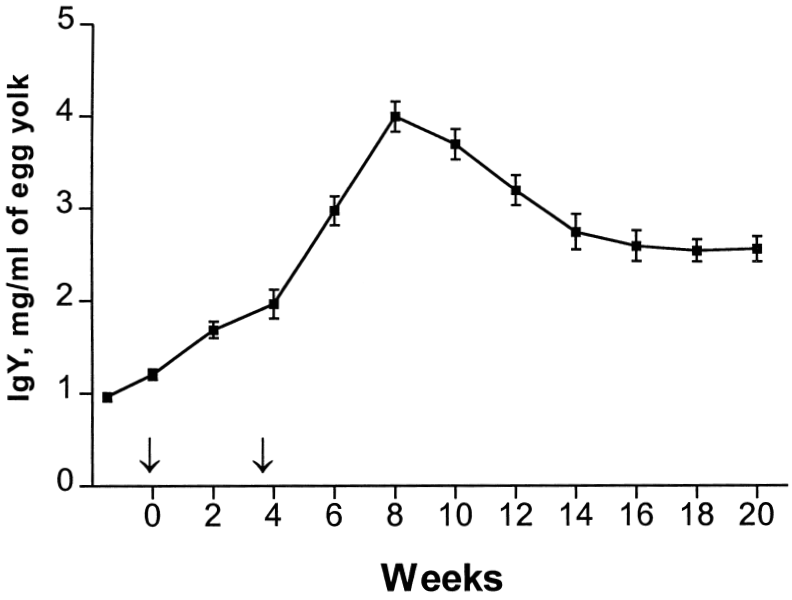


Figure 2. Level of immunoglobulin Y (IgY) content in the samples purified by polyethylene glycol (PEG) 6000. The arrows indicate the time when the hens were immunized with the Sendai virus.

have noted high specific immune responses over 3-15 months in the egg yolk from hens inoculated with various antigens *(Ntakarutimana et al. 1992; Akita & Nakai 1992; Wooley & Landon 1995; Bollen et al. 1995; Bollen & Hau 1998)*.

In the present study, the IgY concentration varied in the range 1.6-4.0 mg/ml of egg yolk throughout the immunization period. Similar levels have been reported by other authors *(Shimizu et al.1988)*, and the increase of the IgY concentration during an immunization constitutes an indirect measure of the efficacy of the protocol used because a significant proportion of the protein in egg yolk is IgY.

From a productivity point of view the yield of IgY obtained by the various purification methods is of interest. Literature reports on the amount of IgY obtained by PEG purification range from 40 to 109 mg/egg (*Hassl & Aspock 1988; Gassmann et al. 1990*). These findings are in accordance with the results of the present work.

Other studies have shown that the of use chloroform gave 7-9 mg IgY/ml of egg yolk *(Verdoliva et al. 2000; Bizhanov & Vyshniauskis 2000)*, which is slightly lower than the 10.7 mg IgY/ml of egg yolk recorded in the present study. Using chloroform, the maximal amount of total protein obtained was 264.0 mg/egg *(Akita & Nakai 1992)*. Chloroform extraction is associated with contamination of the IgY preparation with unwanted nonsense proteins to the extent of 20% in the present study. This presence of many non-IgY proteins has also been demonstrated by SDS-PAGE *(Bizhanov & Vyshniauskis 2000)*.

IgY purification by PEG-6000 resulted in a significantly lower yield of total protein as well as IgY compared with the other purification methods. Interestingly this did not affect the anti-Sendai titre, which was higher than those of the other IgY preparations. The reasons for this are not clear, but it is well known that even low levels of PEG significantly improve immune-complex formation *(Jensenius et al. 1981)*, and this feature has been utilized in precipitation assays like immunoelectrophoresis *(Hau et al. 1981)*. It cannot be ruled out that the IgY purified by PEG still contained PEG molecules which

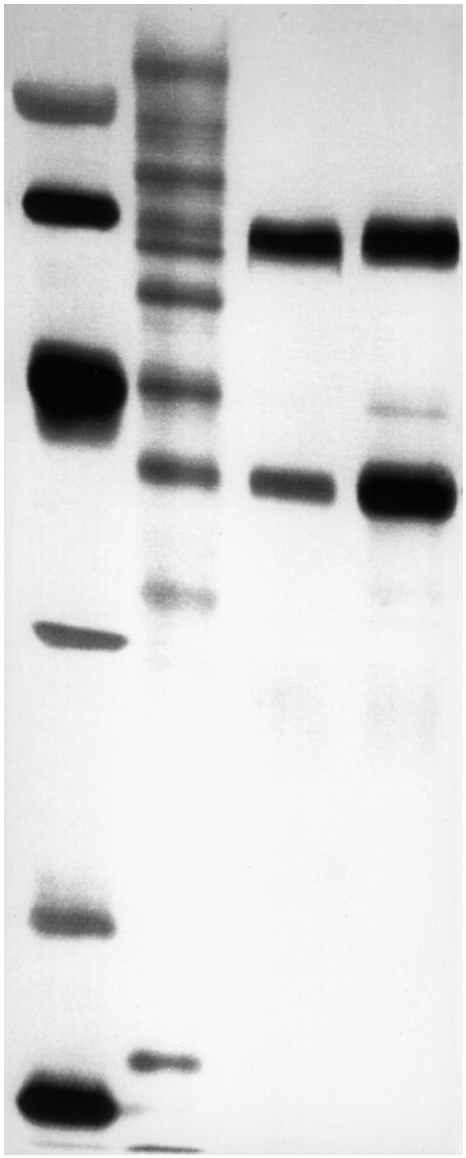

Figure 3. SDS-PAGE of IgY from egg yolk. Each lane was loaded with 5 μg of protein. The acrylamide concentration was 12%. The gel was stained with silver nitrate. Lane 1: molecular weight markers (Pharmacia, Sweden). Lane 2: crude yolk. Lane 3: IgY purified with lithium sulfate. Lane 4: IgY purified with ammonium sulfate.

might have disturbed the haemagglutination assay resulting in abnormally high titre estimations.

Both lithium sulfate and sodium citrate precipitation schemes were found useful for IgY purification. We have previously recorded disappointing impurities when purifying IgY with chloroform, dextran blue and PEG-6000 *(Bizhanov & Vyshniauskis 2000)*. Purity of in particular the IgY purified by lithium sulfate seems very promising when compared with the purity of IgY processed with ammonium sulfate (Figure 3), which is the purification method we use routinely *(Svendsen et al. 1995; Hedlund & Hau 2001)*. A high purity of the IgY preparation is desirable for many immunoassays and for production of labeled second antibodies. It should be noted, however, that for other assay types like many of the immunoelectrophoretic assays high purity is often less important than is monospecificity.

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