V. DISCUSSION

The objective of quality-control works is to characterize the quality of results reported for clinical specimens. This is mostly done by using quality-control specimens, which should reliably indicate the quality of results when they are analyzed in parallel with specimens from patients. Obviously, the quality-control sera must simulate fresh specimens from patients, and this is important because there are different forms of control specimens (freeze-frozen, liquid or lyophilized control serum), produced from different sources (human, bovine or horse). In this study, quality-control serum was prepared from bovine serum which have some matrix different from human serum such as LDH activity is very high whereas cholesterol and triglyceride are very low. However, World Health Organization (Browning, et al., 1986) recommended animal sera rather than human sera because of the serious risk of incorporating of HIV antigen or Hepatitis B antigen into the human control specimen.

Lyophilized control sera are used for quality control programs all over the world because of their prolonged shelf life. It was found that control sera in the abnormal level was always turbid due to the denaturation of cholesterol and lipoprotein which then altered their solubility after reconstitution (Wang, 2000). Denaturation and aggregation of albumin occured during lyophilization was also reported (Lin, et al., 2000). Several sugars or polyols are frequently used as excipients to protect or stabilized protein during freeze-thawing and freeze-drying process (Crowe et al., 1992, 1998, Korey and Schwartz, 1989). Most of recent reports showed that disaccharide should be used as stabilizers or lyoprotectants to protect protein and lipid denaturation. Sampedro, et al. (1998) described effect of carbohydrates to protect plasma membrane H^+-ATPase from *Kluyueromyces lactis* during freeze-during and rehydration. The protective efficiency of carbohydrates was trehalose > maltose > sucrose > glucose > galactose, respectively. In this study, however, four excipients were evaluated for using as lyoprotectants for protein, enzymes and lipid components in quality control specimens. Results demonstrated that after reconstitution, protein and lipoprotein compositions in the control containing saccharose or mannitol were maintained whereas the lyophilized control without addition of any sugar or trehalose showed some changes in protein and enzyme concentrations, which caused by lyophilization. Dextran caused the reconstituted lyophilized sera very turbid as compared with the control and other disaccharides, therefore this sugar was omitted for using as a stabilizer in

this study. Although mannitol showed very good performance in stabilizing protein and lipoprotein during freeze-drying, the effect of crystallinity on the stabilization of enzyme (LDH and β-galactosidase) has been reported (Wang, 2000, Yoshioka, et al., 2000). Moreover, it was found that mannitol tend to phase separate away from protein by crystallizing (Randolph, 1997), therefore from this point of view, saccharose was preferred to mannitol for using as a lyoprotectant of the control sera preparation.

Saccharose is more economically cheaper than mannitol in use as bulking agent for stabilizing protein and lipoprotein which serve to prevent the protein from getting lost during lyophilization cycle and help clarification of reconstituted control sera.

In general, lyophilization increases the turbidity of the reconstituted serum by two to three folds (Kanluan, et al., 1992). This in turn increases the absorbance of the material through most of the visible spectrum thereby interfere with spectrophotometic measurement. Results from this study were agreed with that observation. Adding of saccharose into the prepared liquid control serum resulted in lowering the absorbance of reconstituted control material. Proksch and Bonderman (1976) and our result (Appendix VIII) demonstrated that the turbidity of serum specimen could be measured by scanning the absorbance from 800 to 550 nm. However, a single wavelength at 620 nm was selected to measure the turbidity throughout this study in order to avoid the endogenous yellow color of serum which has an absorbance peak between 570 to 400 nm (Kaplan and Pesce, 1984).

The addition of saccharose in the prepared bovine control sera did not interfere with the analyses of the interest components in control sera and also showed less effect on other components especially for glucose which is a simple carbohydrate important for clinical diagnosis (Appendix IX).

Saccharose is one of the useful bulking agents (Fakes, et al., 2000). Residual moisture contents of lyophilized cakes can be controlled by the use of appropriate bulking agents; althought the moisture content can also possibly be varied by changing the secondary drying cycle during lyophilization. The saccharose in a lyophilized product converted to the amorphous form and had moisture contents not exceed than 2.5% (Fakes, et al., 2000). The average percentage moisture of our prepared products was about 2.02%.

Saccharose, trehalose, mannitol etc. are disaccharides of low molecular weight which known to preserve the native conformation of dried protein during lyophilized preparation (Allison, et al., 1998, 1999).

However, the resulting glass transition temperature (*Tg*, Appendix X) of the sample containing this kind of disaccharide may be too low to ensure adequate storage stability. On the other hand, dextran as high molecular weight carbohydrate, have higher *Tg* but fail to preserve native protein conformation. The optimizing preservation of protein structure and *Tg* of the product was carried out by freeze-drying of protein (actin) in the mixtures of disaccharides and dextran (Allison, et al., 1999). These resulted in increasing formulation *Tg* without affecting the capacity of the sugar to inhibit protein unfolding during lyophilization and thus improved storage stability. In this experiment, the combinations of saccharose or trehalose with dextran to increase *Tg* of the dried control serum formulation compared to those dried with disaccharides alone was also studied. The storage stability was achieved but the reconstituted products were more turbid which means that dextran added in the formulation caused the native protein denaturation. Dextran has various molecular weights (10,000-500,000), lower molecular weight of dextran may be required for our preparation.

The mechanisms of protein stabilization in dry state have been extensively studied, using protein/carbohydrate models, cell and tissues (Sun, et al., 1998). It was observed that direct hydrogen-bonding between dry protein and carbohydrate molecule contributed to the stabilization of labile proteins during air drying and freeze-drying. The soluble carbohydrate could substitute essential water molecule which needed for maintaining the tertiary structure of the protein. The presence of carbohydrates during drying process could maintain proteins in their hydrated conformation.

The other mechanism was proposed for optimal storage stability which requires that lyophilization-induced structural perturbations be minimized and *Tg* be maximized. In practice, these two conditions were difficult to achieve using a single carbohydrate as a stabilizer, because, although *Tg* increases with molecular weight, the capacity of carbohydrates to inhibit lyophilization-induced protein unfolding decreases with increasing molecular weight (Prestrelski, et al., 1995, Tanaka, et al., 1991). As a result, neither protein structure nor *Tg* is optimized in the formulation, and stability may be compromised (Prestrelski, et al., 1995). Allison, et al. (2000) presented that addition of dextran to sucrose or trehalose inhibit protein unfolding during lyophilization and improved storage stability. These results showed that hydrogen bonding between carbohydrate and protein is necessary to prevent dehydration-induced protein damage (Allison, et. al., 1999).

In lyophilization process; temperature, pressure, and sublimation rate were inter-related. As described by Chang and Fischer (1995) in their study of lyophilization cycle development for rhIL-1ra formulated at 100 mg/mL. The experiment was carried out by making comprehensive description of product temperatures and sublimation rates resulting from 35 different combinations of shelf temperature and chamber pressure. They observed the resistance to vapor flow to be independent of product temperature, over a wide range of temperatures (-32 to -14^{0}C). In our study, as saccharose was added in very high concentration (8.5% of liquid control specimen) which then resulted in changing of usual parameter of lyophilized process (Nimsung, et al., 1996-1999), the extending freezing time from 4 to 6 hours, decreasing shelf temperature from –35 to –45 ^{0}C and the product temperature must be kept as low as –30 ^{0}C before continuing the primary drying step.

Enzyme degradation is usually studied by measuring their activities. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis is one of the method use for elucidating the protein inactivation and the stabilized effects of excipients (Lin, et al., 2000). Decrease in alpha globulin fraction in reconstituted control sera observed in this study by SDS-PAGE technique in correlation with decrease in the interest enzyme activity suggested the denaturation of protein to some various degree. Lipoprotein which included total cholesterol was also affected by the lyophilization process. Saccharose in the concentration of 8.5% in control serum preserved the deterioration effect which caused by freezing and drying process during lyophilization.

The shelf life of the lyophilized products was performed by the use of accelerated temperature testing. The guidelines of testing for stability stated that testing should be performed in the actual container intended for marketing (Rockville, 1987). Two accelerated temperature stations were maintained at a constant, controlled relative humidity (RH). In our preparation, the RH was kept constant in vacuum condition of seal preparation, therefore the instability of the lyophilized control products was depended upon the temperature of testing alone. The accelerated temperature testing for determine shelf life of the lyophilized product were performed on both levels of control serum preparation. Results obtained were disagreed between those two levels of preparation. The stability of low level control sera was not accepted because of unstability of AST and ALT observed at 7 days of storage even when kept at 4^{O}C which was the usual temperature for keeping of this product. However, the shelf life reported in this study obtained from the result of accelerated temperature testing on level II control serum. At high levels of chemical

compositions, the change in component concentration on enzyme activities in control serum was caused by the instability of the lyophilized product itself rather than the variability due to the precision of analyses.

In this preparation, it was demonstrated that lyophilization may change pH of the dried protein products. As demonstrated by the depress activities of the aminotransferases in both levels of control sera after reconstitution with distilled water. Reconstitution with bicarbonate diluent was useful for the expression of activities of these enzymes.

From the assessment of these lyophilized bovine control sera, it can be concluded that even the control sera were prepared from pooled bovine sera which the matrix is somewhat differed from that of human serum, all of the experiments were carried out in parallel with using the lyophilized human control serum prepared at the same time of bovine serum preparation as a reference matrix. The lyophilized bovine control sera have potential for use as quality control material for monitoring the precision of analysis of protein, enzymes and lipid components in patients' specimens in clinical chemistry laboratories. The clarity of specimen has no effect on most clinical chemistry tests. The reconstitution of the material was completed within 10 minutes.