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Effects of Storage, Hemolysis, and Freezing and Thawing on Concentrations of Thyroxine, Cortisol, and Insulin in Blood Samples[1] (41466)

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Abstract. Blood samples were collected from six Beagle dogs to determine effects of precentrifugation and postcentrifugation storage times and temperatures, hemolysis, and repeated freezing and thawing on concentrations of thyroxine (T_4), cortisol, and insulin as determined by radioimmunoassay. Concentrations of T_4 did not change in whole and clotted blood stored for 72 hr at 4° or room temperature (22–26°) and in serum stored for 8 days at −20°, 4°, or room temperature. Mean cortisol concentrations decreased ($P < 0.01$) in clotted and heparinized blood stored for 72 hr at room temperature. Mean insulin concentrations decreased ($P < 0.01$) in clotted blood stored for 24 hr and in EDTA-treated and heparinized whole blood stored for 72 hr at room temperature. After centrifugation, mean cortisol concentrations in serum decreased 21% ($P < 0.01$) by 2 days and 57% ($P < 0.01$) by 8 days at room temperature. Insulin decreased 20% ($P < 0.05$) by 2 days and 72% ($P < 0.01$) by 8 days at room temperature. Degradation of both hormones was prevented by storing at 4° and −20°. Hemolysis accelerated disappearance of insulin immunoactivity at 4° as well as room temperature ($P < 0.01$). Repeated freezing and thawing of serum did not affect concentrations of any hormones studied. Our results clearly show that factors such as storage time, storage temperature, and hemolysis changed concentrations of hormones sufficiently to alter interpretation of research and clinical data.

Estimation of concentrations of hormones in blood samples by radioimmunoassay is very useful in endocrine research and diagnosis of endocrine diseases. However, very little has been reported on the stability of hormones in blood samples which are handled and stored under various conditions. Therefore, this study was designed to determine effects of precentrifugation and postcentrifugation storage times and temperatures, hemolysis, and freezing and thawing on concentrations of thyroxine (T_4), cortisol, and insulin in serum and plasma collected from dogs. These three hormones were selected because of their importance in clinical endocrinology and because of their different chemical natures (i.e., an amino acid, a steroid, and a protein).

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Materials and Methods. Adult Beagle dogs weighing 11.4 to 16.2 kg from the Cornell Dog Colony were used in this study. These dogs are known to have relatively low baseline concentrations of T_4, cortisol, and insulin in blood. Decreases in concentrations due to effects of sample handling and storage might have approached the limits of sensitivity of our radioimmunoassays. Therefore, all dogs were treated with thyrotropin (TSH; Jensen-Salsbery Laboratories, Kansas City, MO.) adrenocorticotropin (ACTH; Armour Pharmaceutical Co., Phoenix, Ariz.), and glucagon (Eli Lilly and Co., Indianapolis, Ind.) before blood collection to stimulate maximal concentrations of T_4, cortisol, and insulin in blood. In addition, this hormonal prestimulation was done to minimize effects of stress on concentrations of these hormones when large volumes of blood were collected over a period of several minutes. TSH (5 IU/dog iv), ACTH (2.2 IU/kg im), and glucagon (0.03 mg/kg iv) were

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injected at 4 hr, 2 hr, and 15 min, respectively, before blood samples were collected (1–3).

T_4, cortisol, and insulin were quantified in serum and plasma using previously validated (4, 5) solid-phase radioimmunoassays (Micromedic Systems, Horsham, Pa.).

Experiment 1. In this experiment, we determined if concentrations of the three hormones in plasma and serum were affected by exposure to the cellular elements of whole and clotted blood when stored at two temperatures for various lengths of time before centrifugation. From each of six dogs, 32 ml of blood were drawn by jugular venipuncture into one plastic syringe containing 48 mg of sodium ethylenediaminetetraacetic acid (EDTA), 32 ml were drawn into one syringe containing 480 units of liquid heparin, and 25 ml were drawn into each of two syringes without anticoagulant. The order in which particular syringes were used was randomized for each dog. All four syringes of blood were collected from each dog within 3 to 7 min. Subsamples (2 ml each) of EDTA-treated and heparinized blood were dispensed immediately after collection into sixteen 12 × 75-mm polypropylene tubes, eight of which were kept at room temperature (22° to 26°) and eight at 4°. Subsamples (2 ml each) of blood from one syringe without anticoagulant were dispensed immediately into six polypropylene tubes (clot tubes) held at room temperature and six clot tubes at 4°. Subsamples (2 ml each) of blood from the second syringe without anticoagulant were dispensed into six glass "serum separator" (SS) tubes at 4°. These blood collection tubes have a self-contained serum separator and clot activator (Becton-Dickinson, Rutherford, N.J.). One subsample each of EDTA-treated and heparinized blood was centifuged at 15 and 30 min and 1, 2, 4, 8, 24, 72 hr after collection. Subsamples of blood in clot and SS tubes were centrifuged at 1, 2, 4, 8, 24, and 72 hr after collection. No antibiotic was added to subsamples to prevent bacterial growth. Plasma or serum was recovered from each subsample and stored at −20° until assayed for T_4, cortisol, and insulin. All 56 subsamples of plasma and serum from each dog were analyzed for a particular hormone within a single radioimmunoassay.

Effects of storage times at each temperature on concentrations of hormones were examined by randomized complete block (RCB) analysis with dogs considered as blocks (6). If the F value for treatment (storage time) was significant, Dunnett's test (7) was used to compare mean concentrations in plasma stored 15 min and serum stored 1 hr with concentrations after each subsequent storage time.

Concentrations of hormones in the four types of blood samples were compared by determining the mean concentration for each type of sample over all storage times at 4°. These means were subjected to RCB analysis (6) and Tukey's (7) procedure was used to compare mean concentrations.

Experiment 2. Effects of postcentrifugation storage times and temperatures on concentrations of T_4, cortisol, and insulin in serum were determined in this experiment. Fifty milliliters of blood were collected into a syringe without anticoagulant from each of six dogs and allowed to clot at 4° for 3 hr. Serum was collected and a 1-ml subsample was dispensed into each of 15 polypropylene tubes. Five subsamples were stored at −20°, five at 4°, and five at room temperature. One subsample from each storage temperature was transferred to −70° after 1 hr (0 days) and 2, 4, 6, and 8 days of storage. No antibiotic was added to the subsamples. All 90 subsamples were analyzed within the same radioimmunoassay for each hormone.

RCB analysis (6) was used to determine treatment (days of storage) effects for each storage temperature. Mean concentrations of hormones after 2, 4, 6, and 8 days of storage were compared with the initial concentration (0 days) by Dunnett's test (7).

Experiment 3. Effects of hemolysis on estimation of T_4, cortisol, and insulin were determined in EDTA-treated blood from six dogs. Fifty milliliters of blood were collected by venipuncture from each dog into a syringe containing 75 mg of sodium EDTA. The whole blood was divided into two equal portions. One of these portions was ejected

four times through a 20-ga needle attached to a syringe to produce varying degrees of hemolysis. Two subsamples of this whole blood were removed immediately before ejection through the syringe and needle (0X) and after the first (1X), second (2X), and fourth (4X) ejections. One of each pair of subsamples was stored at 4° and the other at room temperature for 18 hr to simulate overnight storage between collection and centrifugation. The subsamples then were centrifuged and the plasma was stored at −20° until assayed. For each hormone, all samples from five dogs were analyzed together; all subsamples from the sixth dog were analyzed in a second radioimmunoassay.

The entire second 25-ml portion of whole blood from each dog was centrifuged within 1 hr after collection and used for control subsamples. Plasma was recovered, ejected through the syringe and needle, and subsampled in the same manner as was the whole blood. Relative hemolysis of all subsamples was quantified by determining absorbance at 550 nm in a spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

Data were subjected to RCB analysis (6). Mean concentrations of hormones after each ejection of whole blood were compared with mean concentrations in plasma after the corresponding number of ejections (e.g., 4X whole blood vs 4X plasma) by the least significant difference test (7).

Experiment 4. Effects of repeated freezing and thawing on serum concentrations of T_4, cortisol, and insulin were determined in the fourth experiment. Fifty milliliters of blood were collected from each of six dogs and serum was recovered after clotting overnight at 4°. Seven milliliters of serum from each dog were frozen at −20° (2 to 14 hr) and thawed at room temperature (1 to 2 hr) eight times in a single vial. Subsamples were taken after the first (1X), second (2X), fourth (4X), sixth (6X), and eighth (8X) thaws and were stored at 4° until assayed. The remaining serum from each dog was divided into six control subsamples. One of these subsamples (0X) was stored at 4° and the others were frozen. One of these subsamples was thawed simultaneously with each thaw of the large fraction and stored at 4° until assayed 5 days later. Data were subjected to RCB analysis (6) to determine effects of freezing and thawing on each of the hormones.

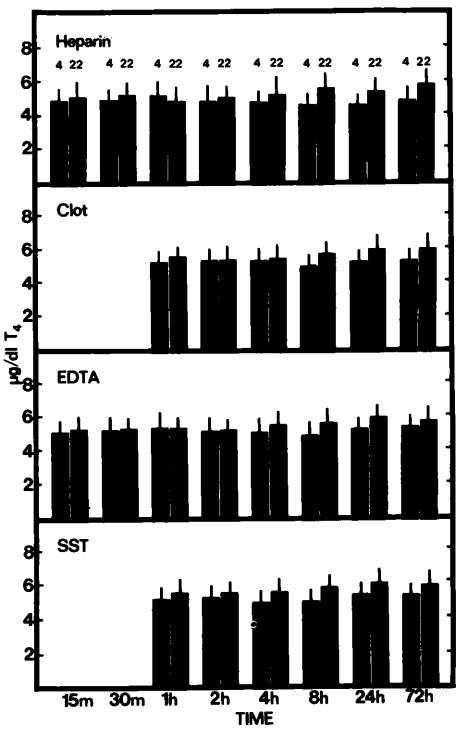

FIG. 1. Concentrations of T_4 ($\bar{X}$ ± SE) in various types of blood samples stored at 4° and room temperature (22°).

Results. *Experiment 1.* Concentrations of T_4, cortisol, and insulin did not change in plasma from whole blood nor in serum from clotted blood after precentrifugation storage for 72 hr at 4°. Plasma and serum T_4 concentrations were also unaffected when blood samples were stored for 72 hr at room temperature (Fig. 1). However, concentrations of cortisol and insulin decreased significantly at room temperature (Figs. 2 and 3). After 72 hr of storage, the mean concentration of cortisol decreased 20% in plasma from heparinized blood and 24% in serum from clot and SS tubes ($P < 0.01$).

Precentrifugation storage for only 24 hr at room temperature caused a 32% reduction in the mean concentration of insulin in

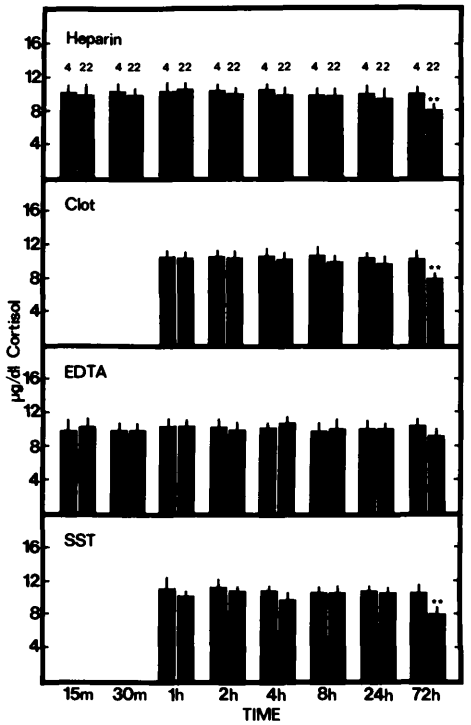

FIG. 2. Concentrations of cortisol ($\bar{X}$ ± SE) in various types of blood samples stored at 4° and room temperature (22°). Significant differences from the initial concentrations are denoted by ** ($P < 0.01$).

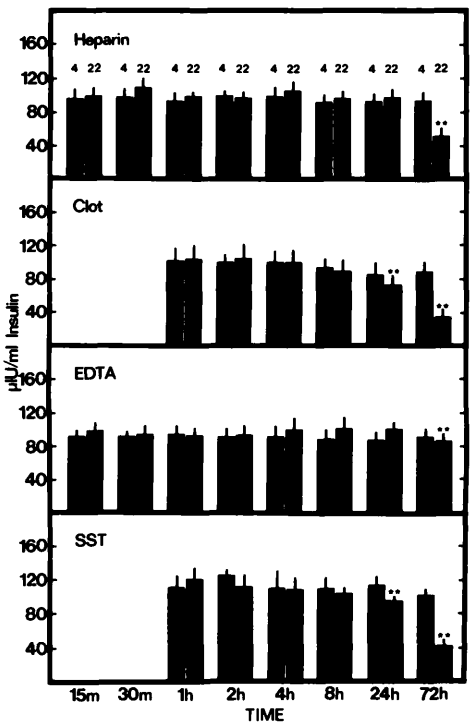

FIG. 3. Concentrations of insulin ($\bar{X}$ ± SE) in various types of blood samples stored at 4° and room temperature (22°). Significant differences from the initial concentrations are denoted by ** ($P < 0.01$).

serum from clot tubes ($P < 0.01$) and a 25% reduction in serum from SS tubes ($P < 0.01$). Storage at room temperature for 72 hr caused a 65% reduction in the mean concentration of insulin in serum from clot and SS tubes, a 43% reduction in plasma from heparinized blood and a 15% reduction in plasma from EDTA-treated blood ($P < 0.01$).

The overall mean concentration of T_4 in plasma from heparinized blood was lower ($P < 0.05$; 4.76 ± 0.62 μg/dl) than that in plasma from EDTA-treated blood (5.20 ± 0.71) and serum from clot (5.17 ± 0.69) and SS tubes (5.22 ± 0.67). The overall mean concentration of insulin was higher ($P < 0.05$) in serum from SS tubes (114 ± 10.60 μIU/ml) than in plasma from heparinized (95.57 ± 9.67) and EDTA-treated (91.27 ± 10.25) blood and serum from clot tubes (95.43 ± 11.80). The mean concentrations of cortisol were the same in all sample types.

Experiment 2. Storage of serum up to 8 days at −20° and 4° after centrifugation did not affect concentrations of any hormone studied (Fig. 4). Concentrations of T_4 did not change even when stored at room temperature for 8 days. On the other hand, cortisol and insulin disappeared dramatically from serum at room temperature. Compared to 1 hr of storage (0 days), the mean concentration of cortisol in serum decreased 21% ($P < 0.01$) by 2 days, 41% ($P < 0.01$) by 4 days, 44% ($P < 0.01$) by 6 days, and 57% ($P < 0.01$) by 8 days. Storing samples at room temperature caused a 20% ($P < 0.05$) reduction in serum insulin by 2 days, a 46% ($P < 0.01$) reduction by 4 days, a 70% ($P < 0.01$) reduction by 6 days, and a 72% ($P < 0.01$) reduction by 8 days.

Experiment 3. Ejecting whole blood through a syringe and needle produced hemolysis in a graded manner as indicated by absorbance at 550 nm (Fig. 5). Significantly less insulin ($P < 0.01$) was measured in

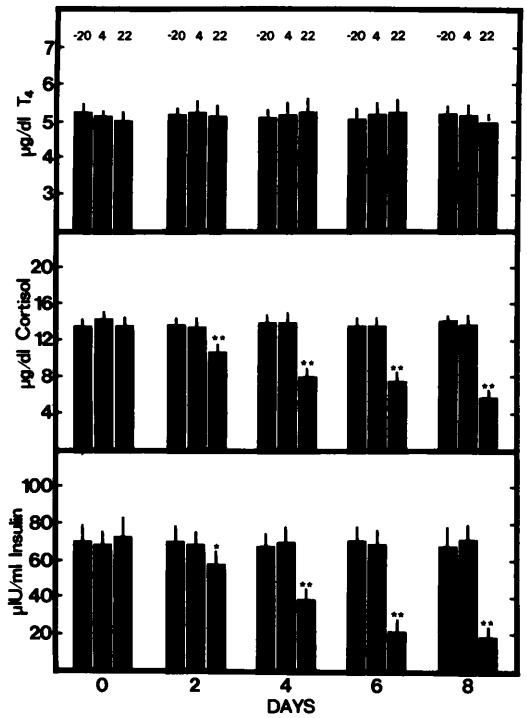

FIG. 4. Concentrations of T_4, cortisol, and insulin in serum stored at −20°, 4°, and room temperature (22°). Significant differences from the initial concentrations are denoted by * ($P < 0.05$) and ** ($P < 0.01$).

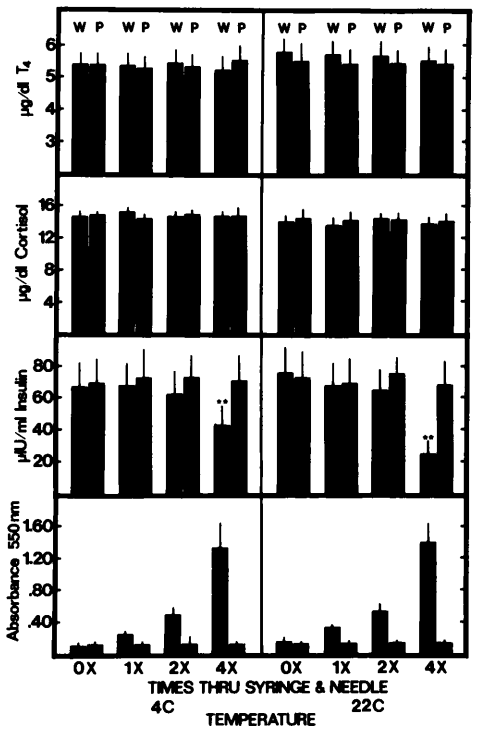

FIG. 5. Concentrations of T_4, cortisol, and insulin ($\bar{X} \pm$ SE) in plasma from whole blood (W) before (0X) and after ejection through a syringe and needle once (1X), twice (2X), and four times (4X) to produce hemolysis. Subsamples were stored for 18 hr at 4° or room temperature (22°) before centrifugation. Significant differences from ejections of plasma (P) are denoted by ** ($P < 0.01$).

plasma from whole blood which had been ejected through the syringe and needle four times than in similarly treated plasma. This effect was seen after storage for 18 hr at 4° (36% reduction) as well as at room temperature (68% reduction). Reductions in concentrations of insulin from samples from individual dogs ranged from 42 to 93%. T_4 and cortisol concentrations were unaffected by hemolysis.

Experiment 4. Freezing and thawing of serum up to eight times did not affect concentrations of any hormone studied (Fig. 6).

Discussion. The results from this study clearly indicate the need for proper storage and handling of blood samples in which hormones are to be quantified by radioimmunoassay. Several reports on differences in concentrations of hormones between plasma and serum have been published (8–11). However, the scarcity of reports on effects of storage temperatures and times, hemolysis, and repeated freezing and thawing is surprising.

In our study, concentrations of T_4 and insulin were influenced by the type of blood sample in which they were measured. Plasma from heparinized blood contained less T_4 and serum from SS tubes contained more insulin than did the other types of samples. Whether these differences and those reported by others were due to actual variability in concentrations of hormones, to interference by anticoagulants, or to assay conditions has not been determined. Although the four types of samples in our study were collected sequentially from each dog, it is unlikely the observed differences in T_4 and insulin were due to factors (e.g., stress) related to sampling technique.

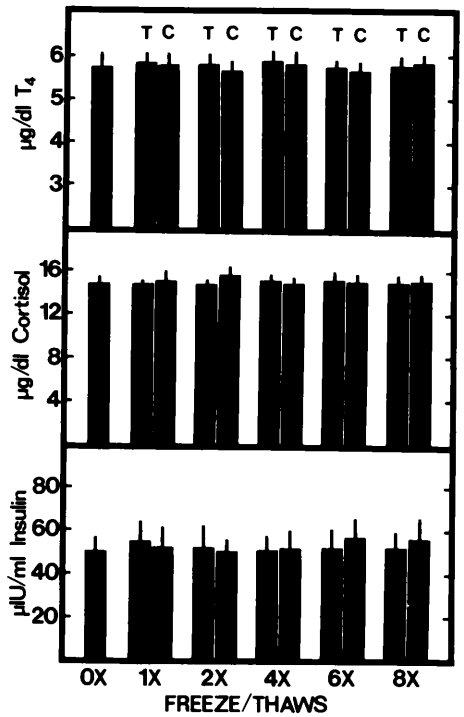

FIG. 6. Concentrations of T_4, cortisol, and insulin ($\bar{X} \pm$ SE) in serum before (0X) and after freezing and thawing one (1X), two (2X), four (4X), six (6X), and eight (8X) times. T, treated (repeatedly frozen and thawed); C, control (frozen and thawed only once).

The order in which the samples were drawn was randomized and all four types were collected from an individual dog within 3 to 7 min. Additionally, the dogs were treated with TSH and glucagon before sampling to stimulate maximal concentrations of T_4 and insulin. Finally, all subsamples of plasma and serum from each dog were analyzed together within a particular assay so that interassay variation could not account for these differences.

T_4 in all types of samples was very resistant to degradation by contact with cellular elements of blood, long-term storage after centrifugation, hemolysis, and repeated freezing and thawing. Serum may be stored for 8 days at room temperature without affecting concentrations of T_4.

Concentrations of cortisol in whole and clotted blood did not change between 15 min and 24 hr when stored at 4° and room temperature. This is contrary to our own studies (unpublished) and previous reports (12, 13) in which concentrations of another steroid, progesterone, decreased rapidly in heparinized, EDTA-treated, and clotted bovine blood. This effect was probably due to contact with blood cells because similar incubation of plasma and serum without cells did not result in decreased progesterone. Erythrocytes are capable of accumulating progesterone and cortisol (14–16) and canine erythrocytes could potentially metabolize both of these steroids because they contain the enzyme, hydroxysteroid dehydrogenase (17). However, no known metabolites of cortisol were detected following incubation of cortisol with canine erythrocytes (15). Thus, the apparent difference in stability between progesterone and cortisol in blood may be due to erythrocytic metabolism of progesterone, but not cortisol. Olson *et al.* (11) also reported no decrease in concentrations of cortisol in clotted and anticoagulated blood stored for 40 hr at 4°.

After centrifugation, cortisol in serum decreased dramatically between 2 and 8 days of storage at room temperature. By 8 days, only 43% of the original cortisol remained. This disappearance could be prevented entirely by storing at 4° and, as expected, by storing frozen.

Insulin was the most labile of the three hormones studied. This is probably related in great part to its proteinaceous nature. Spate *et al.* (18) observed that other blood proteins (glutamic-oxaloacetic transaminase, alkaline phosphatase, lactic dehydrogenase) were much more labile in bovine serum than were glucose, cholesterol, bilirubin, and creatinine. In our experiment, insulin was beginning to disappear from clotted blood as early as 24 hr after collection when stored at room temperature. Insulin seemed to be more stable in both types of plasma than in serum. No decrease in insulin was noted in plasma from heparinized and EDTA-treated blood after 24 hr, whereas, a 25 to 32% decrease was seen in the two types of serum. Furthermore, the decrease after 72 hr at room temperature was considerably greater in serum than in plasma.

There was a significant decrease in serum insulin concentrations by 2 days after centrifugation when stored at room temperature. As with cortisol, merely storing the samples at 4° prevented any loss of insulin immunoactivity. Storing at 4° was as good as storing frozen.

Hemolysis significantly accelerated the disappearance of insulin immunoactivity in plasma. Whereas insulin was unaffected in plasma with minimal or no hemolysis when stored for 72 hr at 4° (Experiment 1), this hormone was significantly reduced in hemolyzed plasma after only 18 hr of storage (Experiment 3). The effect was more pronounced when the samples were stored at room temperature. It should be noted that the degree of hemolysis produced by four ejections through the syringe and needle was not particularly great. These samples were still translucent and the color could be described as brick red. In our experience, this degree of hemolysis is not out of the ordinary and can be expected frequently.

The release of hemoglobin or other contents of erythrocytes (proteolytic enzymes) by hemolysis is known to cause considerable error in many blood chemistry tests (19). This effect may be a direct one on the compound being measured or may be due to nonspecific interference in assay performance. We have not determined if the effects of hemolysis on determination of insulin were due to degradation of the hormone by released intracellular proteolytic enzymes or if the hemoglobin or other components of erythrocytes interferred with our solid-phase radioimmunoassay. The observation that hemolysis exerted its effect both at 4° and room temperature suggests that the effects may be due to nonspecific interference. However, if this were the case, one would expect to measure greater concentrations of insulin in hemolyzed samples, rather than less. Furthermore, the fact that the effect was greater at room temperature than at 4° suggests that the effect is temperature dependent and due to enzymatic degradation. In view of our results, we suggest that research workers doing radioimmunoassays examine their assay systems for effects of hemolysis. Effects on other protein hormones also should be established.

We conclude from our study that improper storage and handling can affect concentrations of hormones in blood samples. Depending on the hormone, factors such as storage time, storage temperature, and hemolysis can change concentrations sufficiently to alter interpretation of research and clinical data.

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