CHAPTER IV

DISCUSSION

Improvement of Methodology.

It is very important to evaluate some preliminary analysis before starting the sensitive and specific RIA of human serum T_4, which almost binds to plasma proteins, especially to thyroxine-binding globulin (TBG). Several radioimmunoassays for T_4 have now been developed employing a variety of compounds to block endogenous TBG. In order to measure serum or plasma T_4 by RIA, ANS was used to displace T_4 from its binding sites and also to prevent binding of added labelled T_4 to TBG for complete reaction between T_4 and T_4-antibody. If ANS was not employed or inadequate, and then estimates of serum T_4 by RIA were 2 to 4-fold higher than competitive protein binding assay (CPBA).[22] The values were even more than 3 to 5-fold when sera of subjects with elevated serum TBG were tested. Furthermore, in absence of ANS, apparent serum T_4 by RIA was indistinguishable between euthyroid and hyperthyroid patients.[22] Therefore, several concentrations of ANS were assessed, and found that 600 ug of ANS per assay tube were optimum concentrations, which was used throughout the period of investigation (see Fig.3, page 28).

In radioimmunoassay, the step of separation bound-hormone from free hormone is particulary important. Desbuquois and Aurbach suggested that antibody-bound hormones were almost completely precipitate from free hormones by polyethylene glycol (PEG), which was inexpensive.[23,24] Thus, a comparison of PEG and charcoal was made and the results showed that PEG gave a good separation, and unlike charcoal, could be added at beginning of the assay and was not time dependent. The optimum concentration of PEG at 15% in final volume was chosen because it gave the best sigmoid curve for T_4 standards on a semi-log graph paper, as indicated in Fig.6, page 31. The equilibrium reaction between antigen and antibody was performed in various time and temperature of incubations for high sensitivity of the assay. Usually the incubation time of routine assay of total serum T_4 is carried out over a period of 16-20 hours at 4°C but the improvement of the present study is obtained by shortening the incubation time from 2 hours at room temperature (23-25°C) to only one hour at 37°C, and no significant difference between two standard curves was found. At these optimum conditions, the best response standard curve was obtained, as showed in Fig.5, page 30.

In order to improve the existing techniques, quality control and cross-reactivity were also analysed to enable accurate assessments of circulating T_4. Intra-and inter-assay variations were evaluated by 15 replications from T_4 standard, diluted with T_4-free serum at low,

medium and high concentrations, which were treated as unknowns to monitor assay performance. The coefficient of variation of inter-assay was found to be greater than intra-assay, as illustrated in Table 1,2, page 32.

The specificity of the assay was determined by cross-reactivity of T_4-antibody to MIT, DIT and L-T_3. No cross-reaction between this T_4-antibody to these compounds was found, but it cross-reacts with L-T_3 at the concentration as high as T_4 (see Fig.7, page 33). It is noted that these hormones (for example L-T_3) are normally present in such low relative concentrations as to render interference with the assay negligible.

Therefore, the simple, sensitive and specific RIA of serum total T_4 was established by several modifications. With these improvements it became possible to finish each assay in one day. Serum total T_4 levels were measured in 219 euthyroid subjects with the mean $\pm$ S.D. of 7.4 $\pm$ 1.6 ugT_4/100 ml, or normal values lay within the range 4.0 to 11.0 ugT_4/100 ml. Satisfactory results were obtained from 25 hypothyroid and 42 hyperthyroid patients, which were well discriminated from the normal values, as shown in Fig.8, page 34. This normal range agrees closely with the experiments of Mitsuma, Nye and Ratcliff.[25-27]

There are many conditions in which abnormallity in thyroid function, so the effects of some drugs on thyroid function have been described since 1947. T_4 levels may be altered by changes in the binding capacity of the thyroid hormones binding proteins. Thus, T_4

levels may be elevated when binding capacity is increased, such as in pregnancy, administration of oral contraceptives or oestrogen preparations, or congenital increases in TBG levels. T_4 levels may be reduced when binding capacity is decreased, such as in congenital decreases in TBG levels, or when drugs which compete for binding sites such as diphenylhydantoin, salicylates or phenylbutazone are administered. These changes result from the fact that the body normally attempts to maintain the concentration of free T_4 at a constant level via various feedback mechanisms. To compensate for the variation in either the amount or the binding effectiveness of serum proteins, the total T_4 concentration is altered. In such cases, assay values for total T_4 will not correlate well with the clinical status, whereas free T_4 values will generally correlate more closely. The direct methods for determination of free thyroxine are time consuming because of its very low concentration and unstability, and none of those methods is applicable to the routine laboratory. This difficulty is commonly overcome by combining the measurement of the total circulating T_4 concentration in serum with that of the concentration of unoccupied thyrobinding sites on the serum proteins (T_3- uptake value). From the results of these two tests a mathematical index proportional to the metabolically active free thyroxine concentration is then derived.[28-32] This "free thyroxine index or FTI" is less affected by abnormallities in the thyrobinding proteins. Thus, FTI values were assessed for interpretation of the effects of

some common used drugs such as acetaminophen, diazepam and nordiol, which are respectively in the group of analgesic, sedative and contraceptive.

Experimental investigations.

Acetaminophen (paracetamol) is now mostly popular because it relieves pain without affecting the alimentary tracts,[33] so the mean ± S.D. of serum T_4, T_3-uptake and FTI before and after 3 gm of acetaminophem therapy for 5 days was compared. Table 3 (page 35.) showed no change between the values of control and treated subjects was found, indicating that acetaminophen may not affect circulating thyroxine and also may not compete or displace endogenous T_4 for TBG binding sites because the values of T_3-uptake and FTI were not changed. It seems reasonable to suggest that acetaminophen may be rapidly removed from the circulation and excreted by tissue cells in the body[6] or the experimental period is short-term treatment that the concentrations of acetaminophen are not enough to alter the levels of total serum T_4. The present findings contrast strikingly with those of other workers, who have shown that analgesic drugs such as aspirin, salicylates, acetylsalicylic acid and indomethacin lower T_4 levels by interfering with T_4-binding to TBG, and in the case of salicylates, binding to the thyroxine binding prealbumin (TBPA) and albumin as well as TBG, resulting in a increase of circulating free thyroxine.[7,34-43]

Diazepam (Valium) is a tranquilizer or sedative drug which has direct effect on the nervous system, suppressing the function of the brain.[44] Therefore, it is interesting to investigate the effects of this drug on thyroid function. The results revealed that only serum T_4 levels were significantly lower ($P \ll 0.001$) in diazepam treatment than in the control but the values of T_3-uptake and FTI were not influenced by diazepam therapy, as indicated in Table 3, page 35. These results suggest that diazepam may influence only total T_4 levels by displacing endogenous T_4 for TBG-binding sites, resulting in a decrease of only total serum T_4 without changing the unsaturated TBG binding sites (UTBG or T_3-uptake test) and also the FTI values. These results are consonant with the evidence that diazepam, barbiturates which are CNS depressants decrease total serum T_4 levels in man and animal.[6,45-51] Another possibility is that diazepam is a tranquilizer which can suppress CNS, involved to hypothalamus area and reduced the amount of TRF and TSH so that T_4 production rate will be decreased.[52-55]

Nordiol is a contraceptive, having less amounts of oestrogen and progesterone. A tablet consists of D-norgestrel 0.25 mg and ethinyl oestradiol 0.05 mg.[56] A great deal of work has been done the effects of contraceptive drugs on circulating thyroid hormones.[5,8,21,57-66] In pregnant women, total serum T_4 levels were

higher than normal non-pregnant[21,59,61] because of increasing of TBG by effects of oestrogen.[57,59-62] This phenomenon was also found in women on oral contraceptives.[61,64,65] The results of this experiment showed that two tested values were increased, (Table 3, page 35), providnig strong evidence that total serum T_4 levels in women on nordiol were significantly higher than controls ($P<0.01$). The values of T_3-uptake were also significantly increased ($P<0.005$) emphasizing the elevation of total TBG concentrations resulting in the increase of UTBG or unoccupied thyrobinding sites on serum proteins. These finding confirm previous suggestion that total serum T_4 is elevated when binding capacity is increased in oral contraceptive administration.[8,61,64,65] It is interesting to note that FTI values do not differ significantly between the two groups (7.5 ± 2.0 cf: $8.2 \pm 1.8\%$, $P<0.1$), indicating that FTI values are not much influenced very much by drug mentioned above, since the free (non-protein-bound) concentrations or FTI of serum T_4 are normal although the total concentrations of T_4 may show quite striking changes. Therefore, it is widely believed that free T_4 exerts biological activity in serum, while it is presumed that the protein-bound moieties are biological inactive as long as they remain bound.

In conclusion, the preliminary investigations showed that FTI may be one of the best indices for routine thyroid function test in vitro because FTI values are not significantly changed in euthyroid subjects which serum proteins (especially TBG) are altered by

oral contraceptive drugs,[57-66] or diazepam (valium) which competes or displaces for T_4-binding sites (Table 3, page 35).

SUMMARY

One of the important parameters of testing thyroid function is RIA, so the sensitive and specific RIA for serum T_4 was developed and improved by several modifications, utilizing 600 ug of ANS per assay tube in barbital buffer as blocker of T_4 binding serum proteins, and PEG at the concentration of 15% in final volume was employed for precipitation of antibody-bound T_4. The simple RIA for total serum T_4 was obtained since the incubation time of the present assay was required only one hour at 37°C. The reproducibility of this assay was excellent in the coefficient of variation of intra-assay was 6.4-8.7% and 8.8-9.7% for the inter-assay variation. The T_4-antibody did not cross-react with MIT, DIT and L-T_3 (at biological concentration) but it slightly cross-reacted with the high concentration of L-T_3. This cross-reaction is unimportant because only small amounts of L-T_3 present in the circulation. All results of these investigations were expressed as a mean $\pm$ S.D., and the values for total serum T_4 in 219 euthyroid subjects were 7.5 $\pm$ 1.6 ugT_4/100 ml. In addition, total T_4 concentration in 42 hyperthyroids and in 25 hypothyroids were found to be 17.6 $\pm$ 4.2 ug% and 2.2 $\pm$ 1.3 ug%, respectively.

Since total serum T_4 levels are influenced by some drugs such as analgesic, tranquilizer and oral contraceptive, the effects of acetaminophen, diazepam and nordiol on the values of total serum T_4, T_3-uptake and FTI were measured and compared between control and treated subjects. The results revealed that acetaminophen (paracetamol) did not affect any values of total serum T_4, T_3-uptake and FTI.

In diazepam treated subjects, the results showed that only total serum T_4 levels were significantly decreased ($P < 0.001$), but the values for T_3-uptake test and FTI were not different significantly from the control group.

The results of treatment of nordiol for a month to 51 healthy women indicated that nordiol significantly increased total serum T_4 levels ($P < 0.01$) and T_3-uptake test ($P < 0.005$) but FTI values ($P < 0.1$) were not significantly increased.

In conclusion, the present study indicate that the free thyroxine index (FTI) was not affected by acetaminophen, diazepam and nordiol, and therefore, the FTI may be solely an excellent indicator of thyroid status.