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Biophysical Study on Thyroxine (T4) and its crude recepters in thyroid tissues

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ABSTRACT

Thyroxine receptors were investigated and measured by radio receptor assay"RRA" in homogenate of Euthyroid, Thyrotoxic and Thyroid carcinoma disease. The results obtained indicated a higher incidence of these receptors in goiter thyroid "i.e. (13.3%) for Euthyroid and(13.64%) for Thyrotoxic" than in tumors tissue "i.e. (10.63%) for Thyroid carcinoma". Spectroscopic studies in the U.V region were carried out on crude thyroxine receptors "CT_4R" and thyroxine hormone "$L\text{-}T_4$". The effects of pH and polarity on the spectra were also studied. Spectrophotometeric pH titration of thyroxine receptors and $L\text{-}T_4$ were carried out and revealed that pKa for histidyl residue was (6.6, 7.7, 6.5) for Euthyroid, Thyrotoxic and Thyroid carcinoma respectively, while the pK_a value of tyrosine residue was (11) for each one of them.

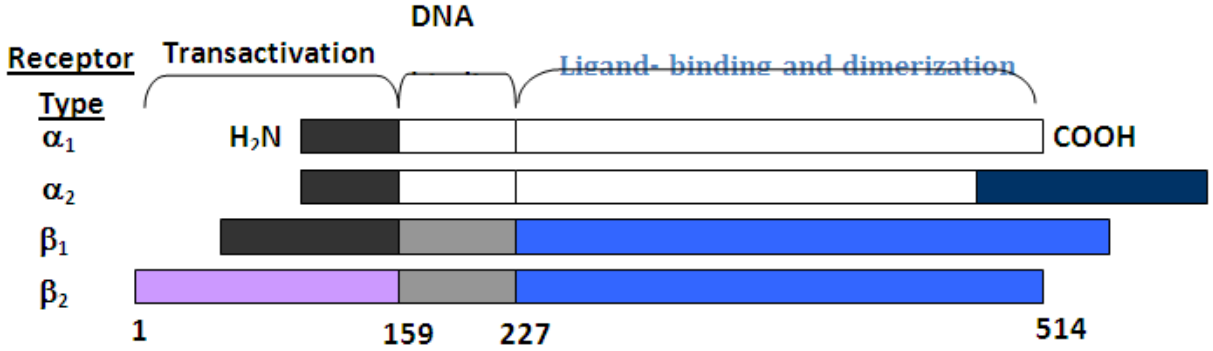
INTODUCTION

Thyroid gland secretes two major hormones Thyroxine (T_4) and triodothyronine T_3 ,which entered into the bloodstream complexed to plasma globulin., causes an increase in the rate of carbohydrate metabolism and arise in the rate of protein synthesis [1,2].

Hormone action begins with the binding of the hormone to its receptor on or in target cell [3]. Receptors for thyroid hormones are members of a large family of nuclear receptors ,their function as hormone-activated transcription factors and thereby act by modulating gene expression. [4]. Hormone binding is associated with a conformational change in the receptor that causes it to function as a transcriptional activator [5].

Mammalian thyroid hormone receptors are encoded by two genes, designated alpha and beta. Further, the primary transcription for each gene can be alternatively spliced, generating different alpha and beta receptor isoforms. Currently, four different thyroid hormone receptors are recognized:

alpha-1, alpha-2, beta-1 and beta-2 as shown below [6].



The different forms of thyroid receptors have patterns of expression that vary by tissue and by developmental stage. The presence of multiple forms of the thyroid hormone receptor, with tissue and stage-dependent differences in their expression, suggests an extraordinary level of complexity in the physiologic effects of thyroid hormone[6,7].

Thyroid disorders are commonly separated into two major categories, hyperthyroidism and hypothyroidism, depending on whether serum thyroid hormone levels are increased or decreased respectively[8,9].

Euthyroid Sick (Euthyroiditis) can be described as abnormal findings on thyroid function tests that occur in the setting of a non thyroidal illness without preexisting hypothalamic–pituitary and thyroid gland dysfunction[10&11]. Diagnosis of euthyroid goiter can be made, TSH levels, estimated free T_4 and antithyroid antibodies must all be normal. Also require a thyroid iodine scan, to attempt to determine hypo or hyperfunctioning nodules[12,13].

Thyroid tumors are the most common endocrine neoplasis [14, 15]. Thyroid carcinoma is a rare condition with an incidence of 0.004% per year according to the third national cancer survey (2001). In Iraq most thyroid carcinoma cases are appear in females, for 1999 and 2000 the percent was (3.03-3%) respectively[16].

Photochemical reactivity is a property of many organic compounds [17,18].In U.V spectral studies ; molecules absorb light the efficiency of absorption depend on both the structure and the environment of the molecule, making absorption spectroscopy a useful tool for characterizing both small and large molecule. The absorption spectrum of a chromophore is primarily determined by the chemical structure of the molecule. However, a large number of environmental factors produce detectable changes in λ_{max} and ε the molar extinction coefficient. Environmental factors consist of temperature, pH, the polarity of solvent or neighboring molecules, and the relative orientation of neighboring chromospheres, these affecters provide the basis for the use of absorption spectroscopy in characterizing macromolecules[19]. In other ward, changes in these environment can lead to alterations in the absorption spectrum[20].

The most common uses of spectroscopic in biochemistry employ the ultraviolet region spectrum[21]. Although it might appear that changes in the U.V absorption of protein side chains could produce only a very limited amount information, the range of studies carried out using ultraviolet absorption proves to be very wide[22]. The aim of the work was investigate the spectroscopic studies on the thyroxin hormone and crude thyroxin receptors in patients with Euthyroid, Thyrotoxi'c and Thyroid carcinoma

EXPERIMENTAL SECTION

Four groups of thyroid disease (72) patients were included in this study.They were admitted from management to several general hospitals including (Baghdad, Al-Kindy and Al-Noor hospitals)

All patients were newly diagnosed and not underwent any type of therapy. Patients suffered from any disease that may interfere with this study were excluded. All surgical operation were down under the supervision of surgeons: Dr. Azaam, Dr. Saab Sdik, Dr. Falah. The host information of all patients were summarized in table (1).

Table (1): The host information of the thyroid disease patients

Groups	Number	Group name	Age (years)
I	22	Euthyroid multinodular goiter	21-57
II	30	Toxic goiter	26-41
III	20	Thyroid carcinoma	40-46
IV	20	Control	19-60

Collection of Specimens and Preparation of Tissue Homogenate:
The enlargement tissues were removal from thyroid gland patients by hysterectomy. The specimens were cut off and immediately rinsed with ice- cold isotonic solution. They were weighted, sliced finely with a scalped then homogenized at (4°C) in (0.01M) tris buffer with a ratio of (1:3) (weight: volume). The homogenate was filtered through several layers of nylon gauze, and then centrifuged at (4000 r.p.m) for (30 min / 4°C). The supernatant obtained represent the cytosolic fraction while the pelt as nuclear fraction. Crude T4 receptors were determined in the cytosolic fraction of each group by Radio Receptors Assay (RRA) [23].

To estimate the U.V Spectrum of crude T_4 receptors in (Euthyroid, Thyrotoxic and Thyroid carcinoma tissues ,a hundred microliters [22.86, 21.88, 22.43 mg protein] of each crude nuclear fraction was completed to (1 ml) with distilled water (pH= 7.3). then placed in a (1 cm) cuvette in sample beam and the absorption spectrum was immediately measured against the adjusted distilled water (pH= 7.3) as a reference.

Study the Factors Affecting on the Absorption Properties of Crude T_4 Receptors in Euthyroid, Thyrotoxic and Thyroid carcinoma Tissues:

pH effect:
A hundred microliters [22.8, 21.8, 22.4 mg protein] of crude receptors were completed to (1 ml)with distilled water at different pH (2.5, 7.3 and 9.5) then each of these mixtures was placed in the test cuvette and the adjusted pH distilled water was placed in the reference cuvette and the absorption spectra of different receptors were measured immediately.

Polarity Effect on the crude T_4 Receptor Spectra :
A hundred micro liters of crude T_4 receptors was completed to (1 ml) with distilled water contains (20%) ethanol or ethylene glycol ,or urea,or DMSO, at (pH= 7.3) then each of this mixture was placed in the test cuvette and the (20%) each solvent adjusted pH was placed in the reference cuvette using (1 cm) cuvette. The absorption spectrum of each sample was measured immediately.

Spectrophotometric pH Titration of Crude T_4 Receptors :
A hundred microliters of crude nuclear fraction were completed to (1 ml) with distilled water at pH rang from (4 - 8). The maximum absorbance of each sample was measured at a wavelength of (211nm). The absorbance of $\lambda_{max\ a}$t each pH value was plotted against the corresponding pH. A nother (100µl) of crude T_4 receptors was completed to (1 ml) with distilled water at pH rang from (9.0-12.5). The maximum absorbance of each sample was measured at a wavelength of (295nm). The absorbance of λ_{max} at each pH value was plotted against the corresponding pH.

Spectroscopic Studies on Thyroxine Hormone: (The U.V Spectrum *):*
A hundred microliters [105 nmol/L] of standard thyroxine hormone purchased from Biosource Company(Europe) was completed to (1 ml) with distilled water (pH= 7.3), then placed in a (1 cm) cuvette in sample beam and the absorption spectrum was immediately measured against the adjusted pH (7.3) distilled water as a reference.

Study the Factors Affecting on the Absorption Properties of Thyroxine Hormones :
pH Effect:
A hundred microliters [105 nmol/L] of standard thyroxine hormone was completed to (1 ml) with distilled water at different pH (2.5, 7.3 and 9.5), then it was placed in the test cuvette and the adjusted pH distilled water was placed in the reference cuvette and the absorption spectra of each sample was measured immediately.

Polarity Effect on Thyroxine Hormone:
A hundred microliters [105nmol/L] of standard thyroxine hormone was completed to (1 ml) with distilled water contains (20%) of ethanol or ethylene glycol ,or urea,or DMSO, at (pH= 7.3) then it was placed in the test cuvette and the (20%) of each solvent adjusted pH was placed in the reference cuvette using (1 cm) cuvette. The absorption spectrum of the sample was measured immediately.

Spectrophotometric pH Titration of Thyroxine Hormone:
A hundred microliters of standard thyroxine hormone was completed to (1 ml) with distilled water at pH rang from (4-8). The maximum absorbance of each sample was measured at a wavelength of (211nm). The absorbance of λ_{max} at each pH value was plotted against the corresponding pH. Another (100µl) of standard thyroxine hormone was completed to (1 ml) with distilled water at pH rang from (9.0-12.5). The maximum absorbance of each sample was measured at a wavelength of (295nm). The absorbance of λ_{max} at each pH value was plotted against the corresponding pH.

RESULTS AND DISCUSSION

The U.V spectra of thyroxine hormone (TH) and crude nuclear T_4 receptors (CT_4R) were measured to determine their maximum wavelengths , and the alteration in the U.V spectra as a results of their interactions.

Figure (1) illustrates the U.V spectrum of (TH); provided by RIA kit, at pH 7.3. The spectrum shows that the λ_{max} is consisted of 2 peaks at 235.6 nm and 272.4nm. As a result (TH) has a characteristic spectrum and can be identified by its peaks, which are assigned to the amide group in the polypeptide bond with contribution of the histidyl residues [21], and tyrosyl residues respectively [19].

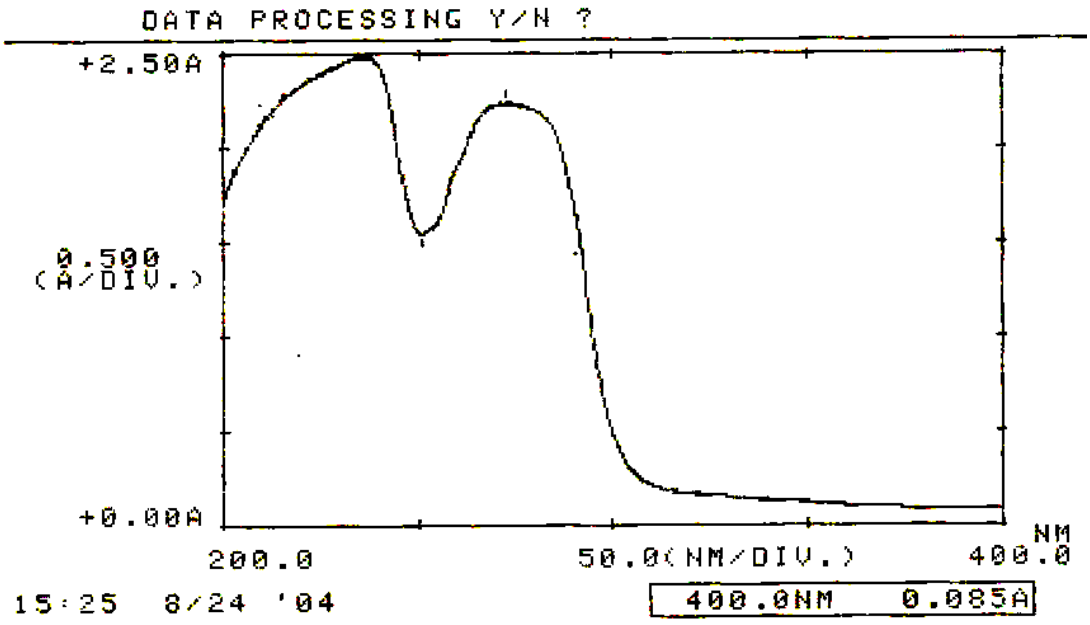


Figure (1) :The U.V spectra of thyroxine hormone

Figure (2) illustrates the U.V spectrums of (CT_4R) for Euthyroid, thyrotoxic and thyroid carcinoma at pH 7.3. As shown the spectrum consisted of two peaks for each one of three groups, a large one at (230.2, 229.2 and 229.4nm) for euthyroid, thyrotoxic and thyroid carcinoma respectively, and as a smaller one near at (277.6, 277.6 and 276.1/nm) for euthyroid, thyrotoxic and thyroid carcinoma respectively. As a result euthyroid, thyrotoxic and thyroid carcinoma have a characteristic spectrum and can be identified by their peaks, the first peak (230.2, 229.2 and 229.4nm) due to the amid group in the polypeptide bond of (CT_4R) molecule [21]. While the second peak at

(277.6, 277.6 and 276.1nm) are assigned to the side chain chromosphere of tyrosyl residues The data in table (2) illustrated the values of λ_{max} for U.V spectra of TH and CT_4R

Table (2): The values of λ_{max} for U.V spectra of TH and CT_4R

Samples	λ_{max1}, λ_{max2} (nm)
(1) Thyroxine hormone	235.6, 272.4
(2a) Euthyroid tissue	230.2, 277.6
(2b) Thyrotoxic tissue	229.2, 277.6
(2c) Thyroid carcinoma	229.4, 276.1

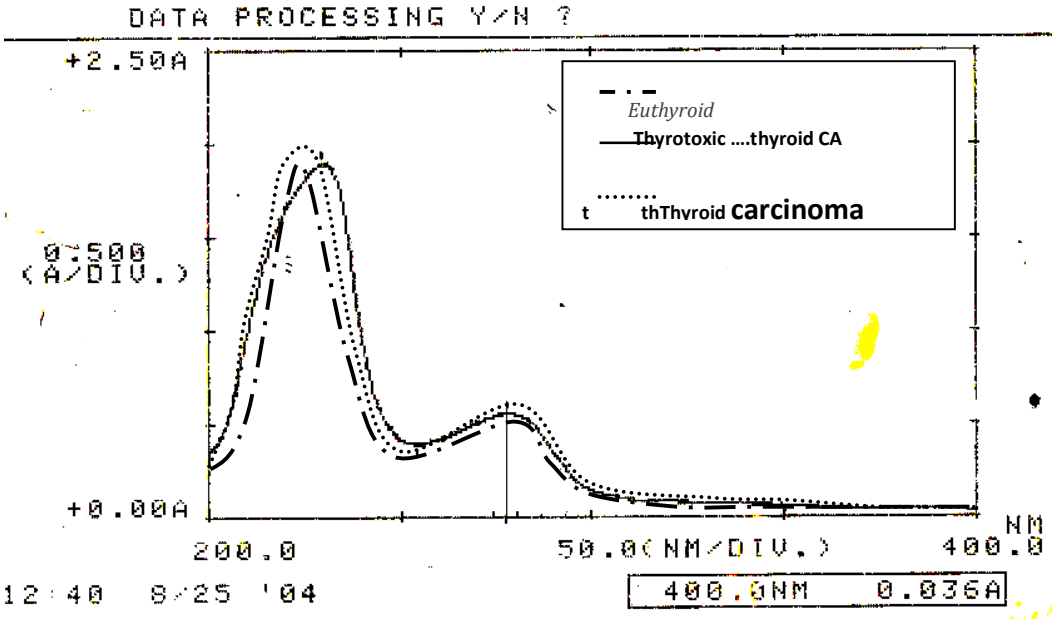


Figure (2): The U.V spectra of crude T_4 receptor:(a) Euthyroid, (b) Thyrotoxic, (c) Thyroid carcinoma.

Factors Affecting the Absorption Properties :

The absorption spectrum of chromophore is primarily determined by the chemical structure of the molecule.). Middendorf andAldrich ,2000) used UV modification to assess the role of aromatic residues incyclic nucleotide-gated ion channels(24). However, a large number of environmental factors produce deflectable changes in λ_{max} .Environmental factors consist of pH, the polarity of the solvent or neighboring molecules [(19)].

pH Effect:

The pH of the solvent determines the ionization state of the ionizable chromophore in the protein molecule [(19)].

The U.V spectrums of (TH) and (CT_4R) were determined at the pH's (2.5, 7.3, 9.5). Figure (3 : a, b, c, d) shows these spectrums respectively.

It seems that in acidic region at pH 2.5 there were a blue shift in λ_{max} . As shown in figure (3 : a, b, c, d) the λ_{max} , in (TH) shifted to 234.2nm while in(C_4R for euthyroid, thyrotoxic and thyroid carcinoma shifted to (226.4, 225.0 and 228.0nm) respectively, these blue shift are due to increasing the intra hydrogen bonds formed in the presence of highly positively charged state[(20)].

These results are in agreement with Laskowsk, et al., (1960) observations[(25)]. On the other hand, in the same medium pH 2.5 λ_{max2} in (TH) shifted to 276.8 nm, this red shift in the protein spectrum which produced by decreasing pH cannot be simply attributed to the inductive effect at vicinal charges, such spectral changes must therefore attributed

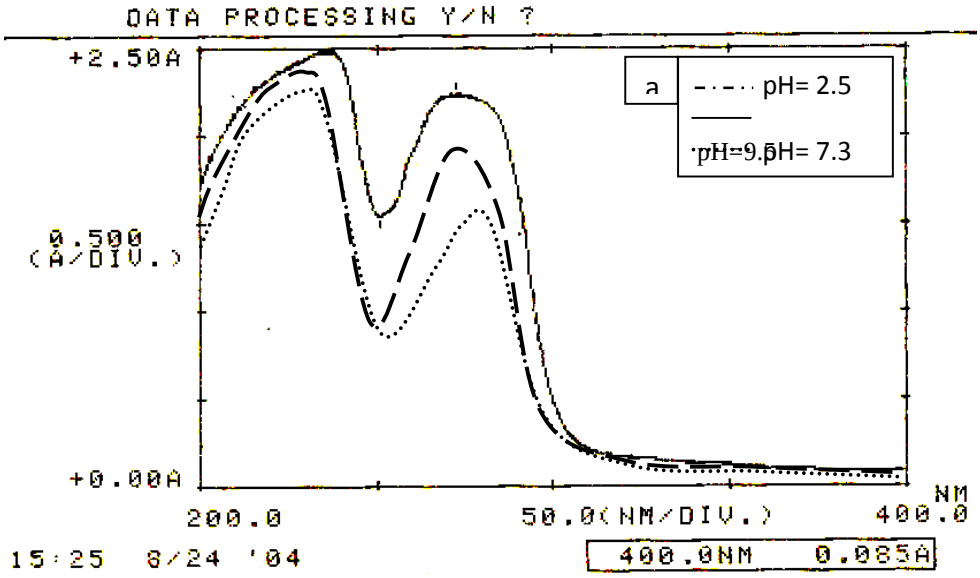
mainly to rearrangement of secondary and tertiary structure, although the possibility of field effects due to unusually close conjunction of charges of aromatic groups is not excluded [(26)]. The studies of Yang and Foster[(27)] and Tranford et al., [(28)] have also revealed that as pH as lowered below 4.3, the protein undergoes a fairly sharp conformational change and increasing the flexibility of the protein on further acidification.

Also, λ_{max2} in CT_4R for euthyroid, thyrotoxic and thyroid carcinoma spectrums shifted to shorter wave length (276.4, 276,275nm) respectively. These blue shifts assigned to conformational change in the protein[(21)]. When the pH was increased to 9.5 there was a significant shift to a shorter wavelength (blue shift) for λ_{max1} in (TH) this shift may be due to the increasing in the intra hydrogen bond that is formed between the molecules of TH itself [(29)]. In CT_4R molecule for euthyroid, thyrotoxic and thyroid carcinoma spectrums, there were a significant shift to longer wave length (red shift) for the polypeptide bond λ_{max1} (231,230.2,230.4nm) respectively. This is due to the hydrogen bonding of the amid group of polypeptide bond with alkaline solution as a hydrogen acceptor[(30)].

These increments were associated with increasing λ_{max2} to 278.2, 279.0, 28 and 280nm) in TH and CT_4R for the three groups spectrums respectively. This red shift is assigned to appeared tryptophyl residues on the surface and buried tyrosyl residues inside the protein molecules due to a conformational change in the molecule. Table (3) show the effect of pH on the λ_{max} of TH and CT_4R U.V spectrums

Table (3): The effect of pH on the λ_{max} of TH and CT_4R U.V spectrums

pH	λ_{max1} , λ_{max2} (nm)			
	Thyroxin hormone	*euthyroid*	*thyrotoxic*	*Thyroid carcinoma*
2.5	234.2,276.8	226.4,276.4	228,276	228,275
7.3	235.6,277.4	230.0,277.6	229.2,277.6	229.4,276.2
9.5	243.2,278.2	231,279	230.2,280	230.4,280



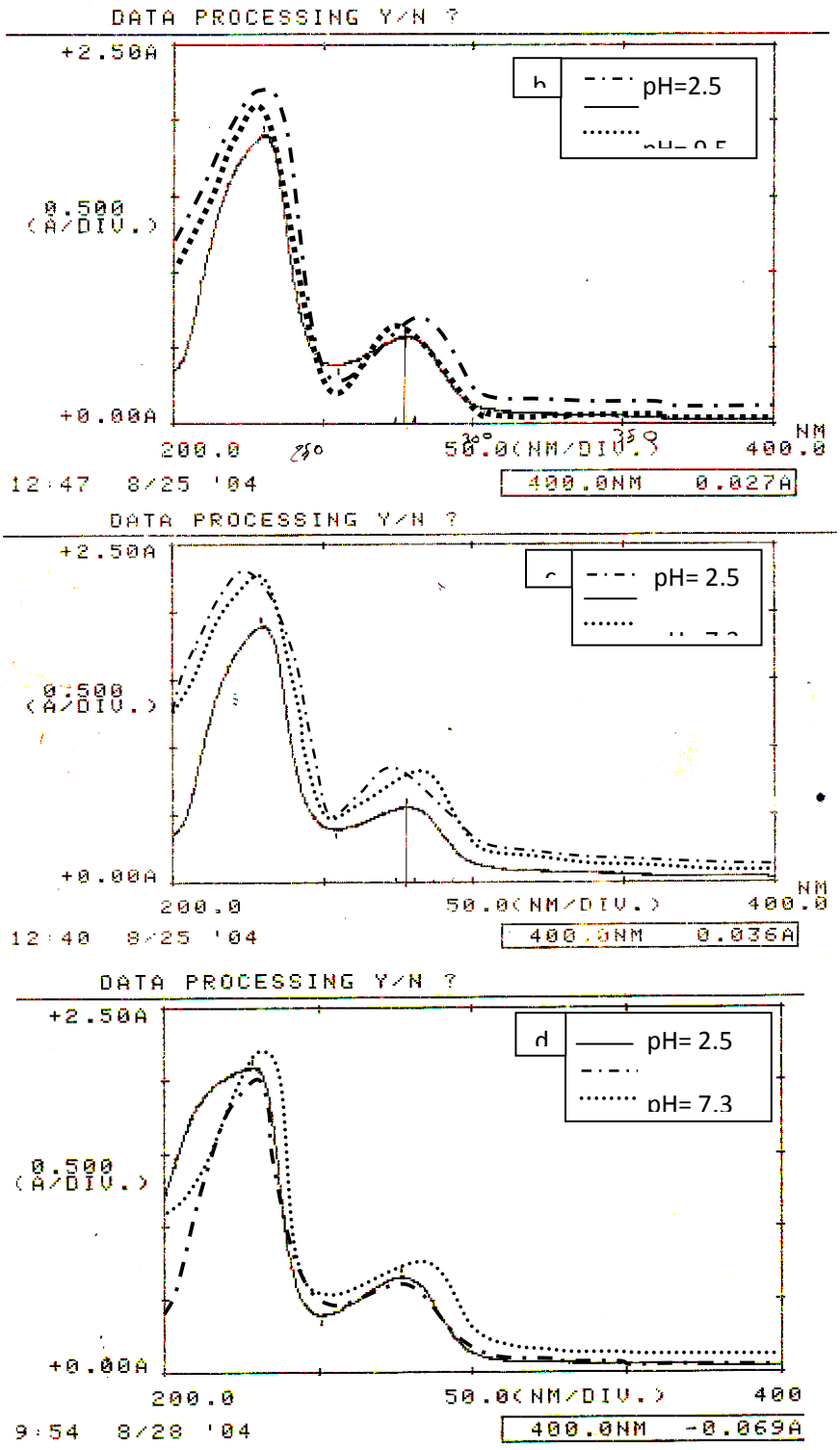
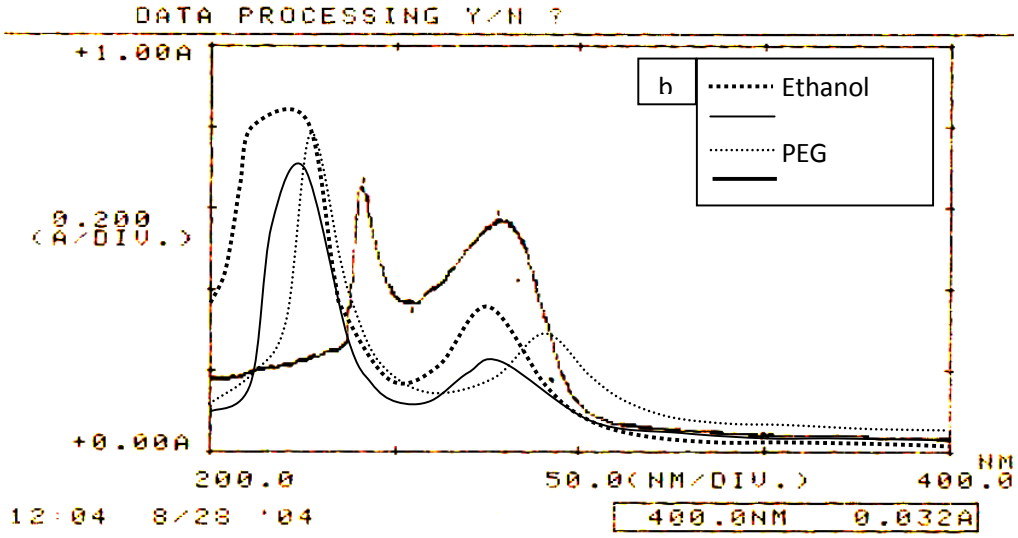


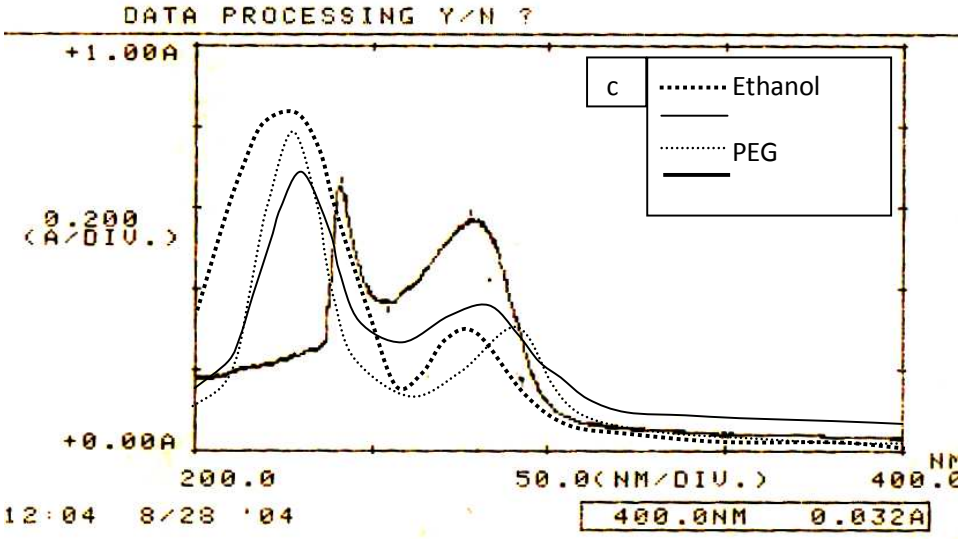
Figure (3): The effect of pH on U.V spectrum of:(a) Thyroxin hormone (b) Euthyroid receptors, c)Thyrotoxic receptors, (d) Thyroid carcinoma receptors.

Polarity Effect on: UV Thyroxine Hormone and UV Crude T_4 Receptors

The immediate environment of a chromophor affects its absorption. The determination of whether an amino acid is internal or external by measuring the spectra of protein in a polar and non polar solvent is called the solvent perturbation method [31].

The Effect of 20% Ethanol: Table (4) and figure (4 a, b, c, d) show the effect of 20% ethanol at nature pH. The λ_{max1} values of polypeptide bond and histdyl residues were shifted towards shorter wavelength (blue shift) in 20% ethanol for (TH) and (CT_4R) for euthyroid, thyrotoxic and thyroid carcinoma respectively. This slight decrease in the absorbency of histidyl could be attributed to change in the protein structure, that histidyl residues were partly embedded in a hydrophobic region of the protein molecule. Also, the λ_{max2} values of tyrosyl residues were shifted towards longer wavelengths (red shift) due to the inter hydrogen bonding of OH groups of tyrosyl residue with the solvent, where tyrosine was functioned as a hydrogen donor [26,30].





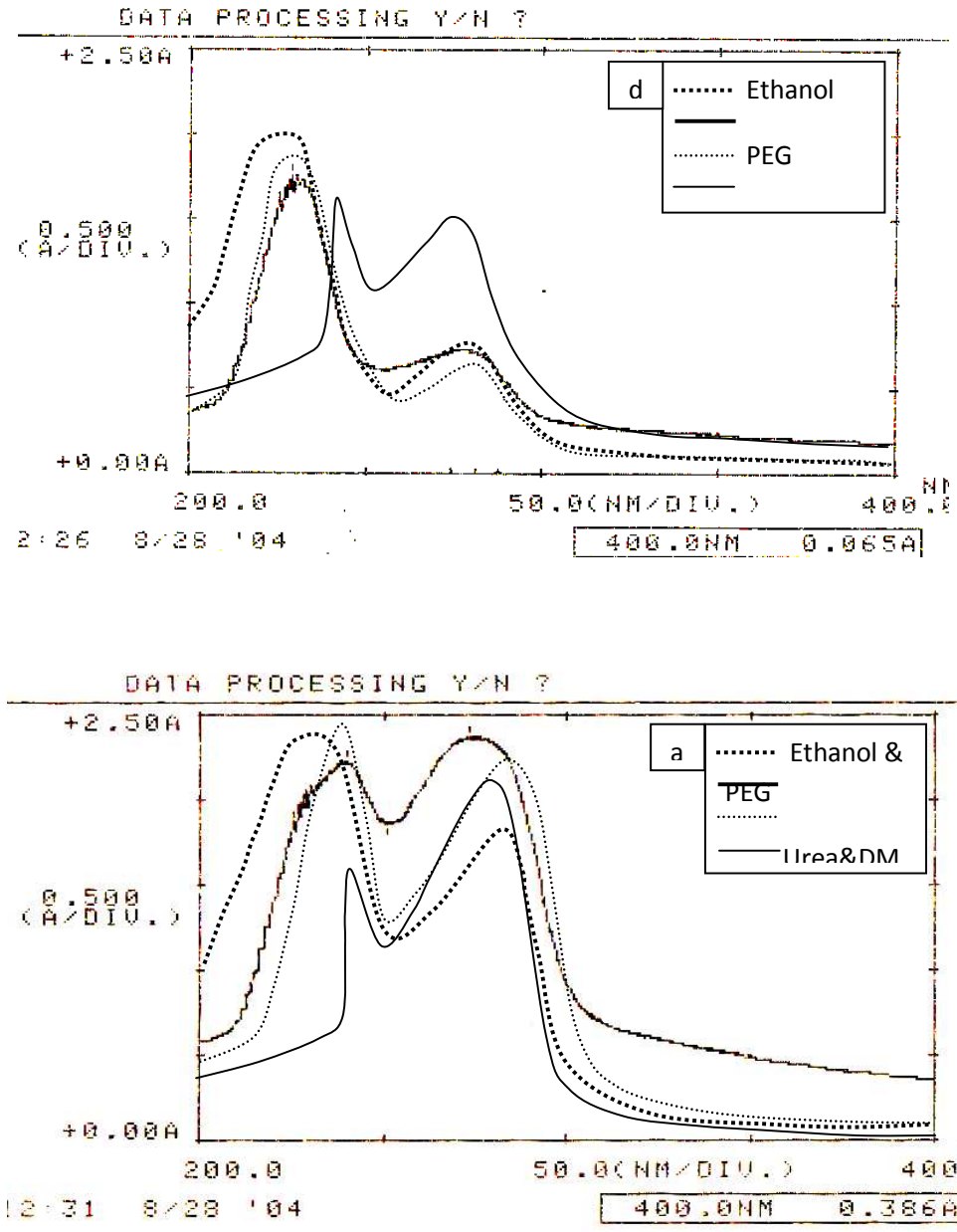


Figure (4): The difference spectra obtained at neutral pH 7.3 in: (a) Thyroxin hormone (b) Euthyroid receptors, (c) Thyrotoxic receptors, (d) Thyroid carcinoma receptors with different solvents.

Table (4): The effect of 20% of ethanol, polyethylene glycol (PEG), dimethylslphoxid and urea.

Solvent	λ_{max1}, λ_{max2} (nm)			
	TH	*Euthyroid*	*Thyrotoxic*	*Thyroid carcinoma*
(20%) EtOH	234.4, 277.8	228.6, 277.2	228.2, 278	228.6, 277.2
(20%) PEG	240, 273.2	230.2, 278	231.8, 279	228.6, 278
(20%) Urea	237.2, 279.6	230.4, 282	230.8, 281.4	230.4, 280.4
(20%) DMSO	242.2, 276.2	241.2, 279.6	241.2, 278.6	241.2, 278

The effect of 20% polyethylene glycol:

Table (4) and figure (4: a, b, c, d) show the effect of 20% polyethylene glycol at neutral pH. The λ_{max1}values of polypeptide bond for TH and CT_4R in thyroid homogenate were shifted towards longer wavelength (red shift) in presence of 20% polyethylene glycol. These alteration in the positions at λ_{max1} are all due to the intermolecular hydrogen bonding between amide group in the TH or CT_4R molecules with the poly OH solvent (i.e. PEG) . The intermolecular hydrogen bonding increase as the concentration of solution increase and may be additional bonds start appear at longer or shorter wave length[29], excepting in CT_4R for thyroid carcinoma spectrum. There was a slight decrease in the absorbency of λ_{max1} it could be attributed to a change in the protein structure.

While λ_{max2} values for TH and CT_4R for euthyroid, thyrotoxic and thyroid carcinoma were shifted to a longer wave length (red shift); table (4) shows these shifted caused by presence of the inter hydrogen bond between the -OH groups of tyrosyl residues with the solvent PEG, were tyrosyl was function as a H^+ donor [30,32], excepting in CT_4R for thyrotoxic spectrum the red shift in λ_{max2} is due to tryptophyl residues.

The Effect of 20% Urea:

Table (4) and figure (4: a, b, c, d) show the effect of 20% urea at natural pH on TH and CT_4R. The values of λ_{max1} were shifted to a longer wavelength (red shift) due to the hydrogen bond between amid group and the solvent (urea), while the significant red shift for λ_{max2} is due to a new chromophore appeared (tryptophyl residues) in presence of 20% urea.

The appearance of this new λ_{max} value indicates that the protein was defolded due to change in the secondary and tertiary structure of the protein that bring the tryptophan to expose to absorbance, while tyrosine residues were buried inside the hormone and receptor molecules, also it was found that TH and CT_4R are highly sensitive to change in the polarity of solvent.

The Effect of 20% DMSO:

Table (4) and figure (4: a, b, c, d) show the effect of 20% DMSO on TH and CT_4R U.V spectra at pH 7.3 comparing the λ_{max1} value for TH with there was a assigned increase (red shift) in the absorbency due to the inter hydrogen bonding between the amid groups and the solvent (DMSO), while the red shifted λ_{max1} of CT_4R for the three groups indicate to the appearance histidyl residues on the surface of the protein molecules. Unfortunately, it will be difficult to detect histidyl difference spectra in protein because of it's high absorbance occurred in the low wavelength region of contributed with the peptide bond absorbance, therefore high difference of spectrum is caused by tyrosine and tryptophan[20].

The values of λ_{max2} for TH and CT_4R for thyrotoxic and thyroid carcinoma were shifted to a longer wavelength; these red shift due to the hydrogen bonding between the OH group of tyrosyl residues and the solvent molecules (DMSO); the tyrosin played a H^+ donor role [30,32], excepting in CT_4R for euthyroid spectrum there was a red shift in λ_{max2} caused by the appearance of tryptophyl residues.

Spectrophotometric pH titration of:

Thyroxine hormone and crude thyroxine receptors in:Euthyroid, Thryotoxic and Thyroid carcinoma tissues*:*

Spectrophotometric pH titration is the following of the change in absorbance with pH as alkali (or acid) added[31]. This can only be done when dissociation of an acid group involves a spectroscopic change in the chromophor.

For protein this usually occur when phenolic group of tyrosine residues, is titrated by measurement of tyrosine residues, and following the absorption at 295nm (λ_{max} for ionized from of tyrosine)), or observation of histidine dissociation by measurement at 211nm.

To study TH and CT_4R structures, this require the determination of pKa value for protein dissociation from ionzable amino acid side chains, because these values give indication of the location of the amino acid in the protein.

Figures (5: a, b, c, d, e, f, g, h) show the pH titration curve of (CT_4R) and (TH) for histidine and tyrosine respectively, the curves a, b, c, d show that pK_a values for histidine are [6.6.,7,7,6.5] for TH, euthyroid, thyrothyrotoxic and thyroid carcinoma respectively, while the pKa value for tyrosine in curves (5: e, f, g, h) are equal to (11) for each one of them[31].

In both figure (5: a, b, c, d and e, f, g, h) the spectral changes as a function of pH for the ionizable groups (i.e. the OH tyrosine, imidazole of histidine) indicates a different pka from that of exposed group then, the amino acids tyrosin and histidine in both TH and CT_4R molecules are likely to be on the surface of the molecules.

On the other hand, the figure (5: a, d) the spectral changes as a function of pH have the same pKa as it would be if they were free in solution then the histidine in TH and CT_4R for thyroid carcinoma are located on the surface of protein.

A large rise in the absorbance at very high pH was observed figure (5: e, f, g, h). This indicates that the internal histidine and tyrosine have become exposed to the solvent, that is the protein TH, CT_4R become unfolded and become denatarated.

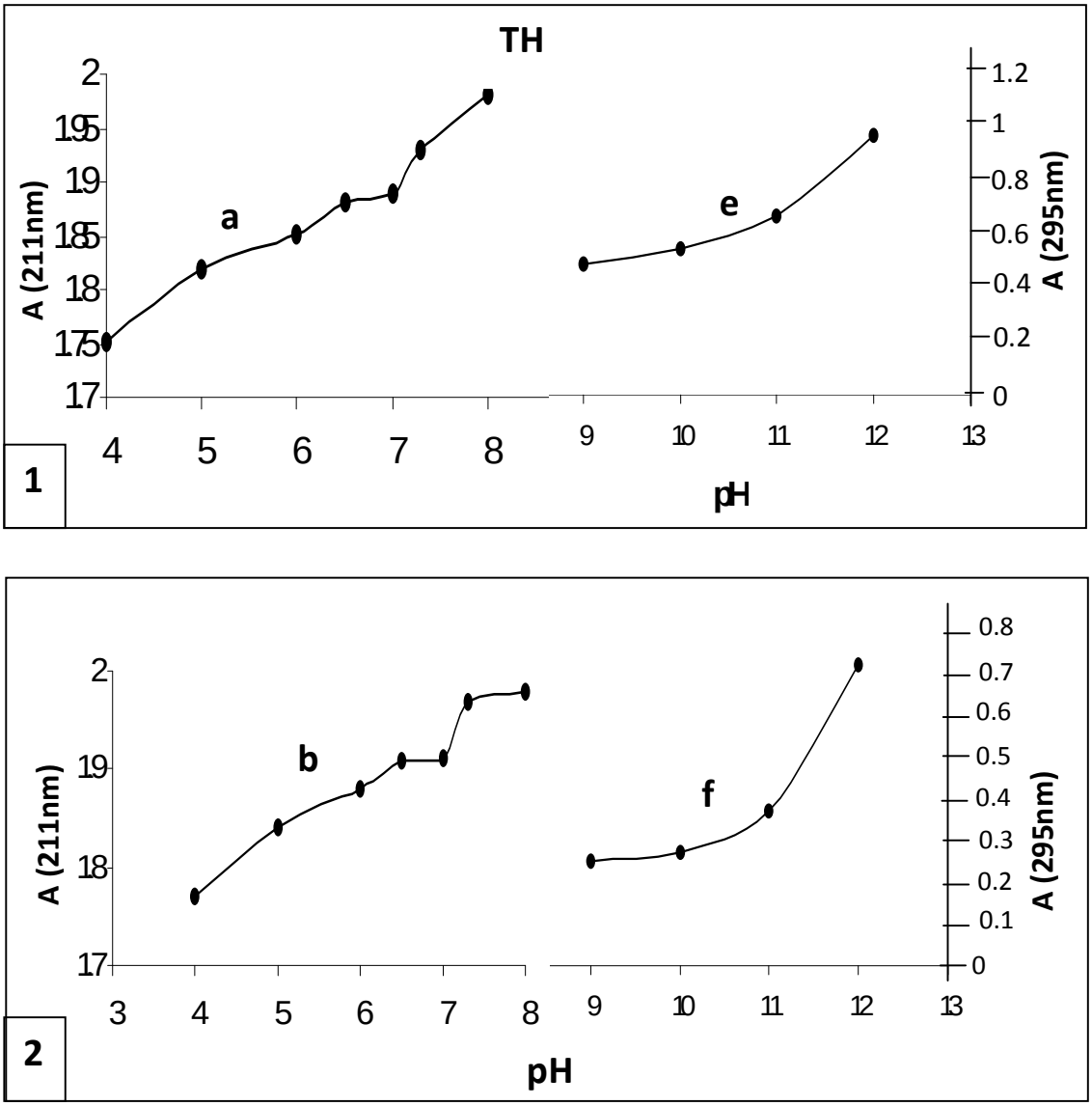
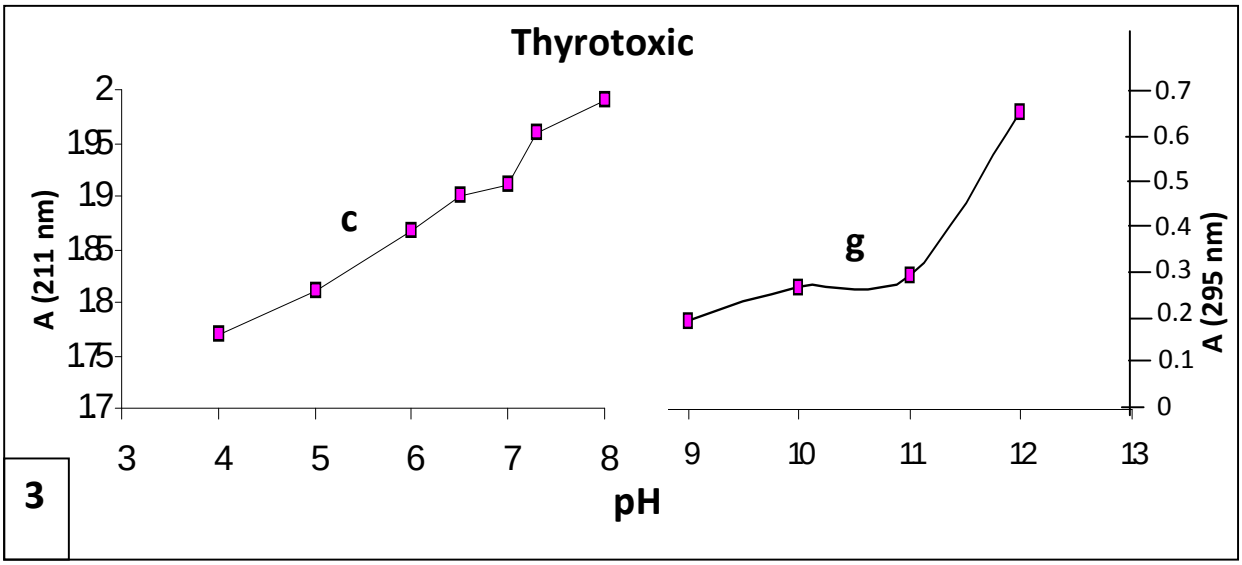


Figure (5): Spectrophotometric pH titration of: (1) Thyroxine hormone TH, (2) Euthyroid receptors .



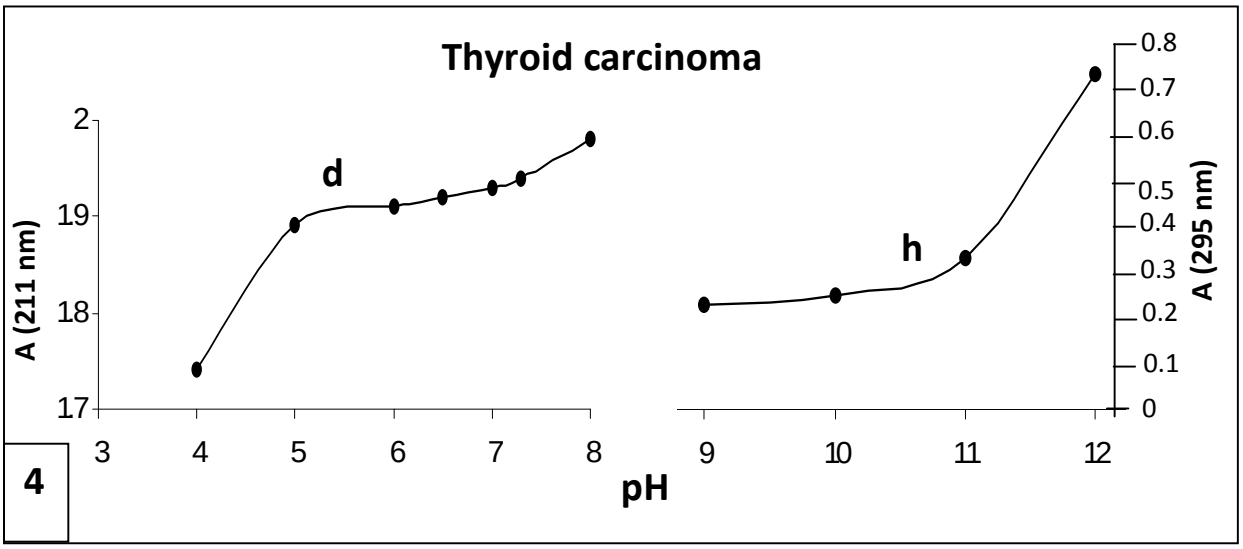


Figure (5): Spectrophotometric pH receptors .titration of: (3) Thyrotoxic receptors & (4) Thyroid carcinoma

CONCLUSION

A higher incidence of thyroxin receptors was obtained in thyroid goiters "i.e. Euthyroid and Thyrotoxic" than in Thyroid carcinoma, therefore the thyroid goiters were more thyroxin dependent than those of thyroid tumors. The spectroscopic studies on crude thyroxine receptor and thyroxin revealed a characteristic spectrum for each receptor and the hormone "L-T_4".

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