SPECTROPHOTOMETRY OF THE PERIODIC ACID–SCHIFF REACTION WITH PITUITARY HORMONES *IN VITRO* AND IN HISTOLOGICAL SECTIONS

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ABSTRACT

The spectral light absorption of the *in vitro* periodic acid–Schiff reactions of 4 purified pituitary hormones is described. The absorption spectra present a maximum between 560 and 565 mμ. The color developed conforms with Beer's law for the ranges of concentration examined. The different hormones exhibit different chromogenicity per unit of biological activity: the color produced by 1 unit of FSH is equivalent to approximately 2 of TSH, 4 of LH, and 30 of ACTH. Microspectrophotometric measurements of the PAS-positive structures in histological sections of the human pituitary give absorption curves with shapes similar to those obtained *in vitro*, although quantitative differences exist. It is concluded that under the proper experimental conditions microspectra of the pituitary structures might, in the future, prove to give a quantitative measure of aldehyde groups generated from glycoprotein tropins by periodate oxidation.

The periodic acid–Schiff (PAS) reaction (15) is the basis of several differential staining methods for typing pituitary cells (1, 6, 18, 22); the glycoprotein tropins follicle-stimulating hormone (FSH), luteinizing hormone (LH), and thyrotropic hormone (TSH) have been demonstrated within pituitary basophils by the pink to red color characteristic of PAS-positive sites (3, 10). The periodate oxidation cleaves α-glycols and certain related groups in the hormone molecules to aldehydes which then form colored products with the Schiff reagent. Although the chemistry of the Schiff-aldehyde reaction is not yet fully understood, it is feasible, under certain conditions, to use it for quantitative colorimetry. An extensive review of this subject has been published by Kasten (9).

The isolation of purified preparations of anterior pituitary hormones makes it possible to determine, *in vitro*, their chromogenic properties in the PAS reaction under conditions similar to those applied histochemically. The spectral characteristics of the colored reaction products of the hormones can then be applied to assess the PAS staining of human pituitary cells in tissue sections by microspectrophotometry.

It has been shown that the Schiff-aldehyde reaction proceeds in steps (2, 8) to yield several, slightly different, colored compounds. The various proportions between these compounds depend on *inter alia* the time of reaction and the quantitative relationship between aldehyde groups and reagents Thus, different shapes of spectral absorption curves might arise and, provided that the measurements are of sufficient accuracy, some qualitative distinctions might be expected in addition to the quantitative chromogenic effects. Developments of the microspectrophotometric technique in the past

decade (4, 9, 20, 21, 23) give enough sensitivity and/or stability for accurately recording absorbancy changes of 0.1 to 1.0 per cent from cell areas of 1.5 μ in diameter. Small differences in the shape of absorption curves, therefore, can be detected also in measurements of the cytological structures of the pituitary.

The present paper describes some spectrophotometric properties of the reactions *in vitro* between Schiff reagent and the 3 glycoprotein hormones after their oxidation with periodate, and also the PAS reaction of the periodate-oxidizable *n*-terminal α-amino alcohols occurring in several of the other tropins (12, 13). By means of microspectrophotometry (23) the *in vitro* results are compared with the PAS reaction in histological sections of the normal human pituitary.

TABLE I

*Identification and Characteristics of the Pituitary Hormones Studied**

Substance	Species	Biological activity of preparation	Purity of preparation	Method of ascertaining purity
		per mg.		
FSH	Pig	1 Armour u.	85%	Chromatography
TSH	Cow	1 USP u.	65%	—
LH	Horse	4.5 Armour u.	90%	Chromatography
ACTH	Pig	30 USP u.	20%	XE 97 Chromatography

* We would like to acknowledge our indebtedness to Dr. Joseph D. Fisher of the Armour Pharmaceutical Company, Kankakee, Illinois, who supplied generous samples of these hormones and provided the information summarized here. Other preparations of some of these hormones were kindly supplied by Dr. Fredric Paulsen of Ferring AB, Malmo, Sweden.

MATERIAL AND METHODS

Substances Examined

Table I presents data on the species of origin, activity on bio-assay, purity and the method of ascertaining purity of the hormone preparations used. Since the reactive groups in the glycoprotein hormones are hexose units, analytically pure samples of glucose, galactose, and mannose were also examined. Furthermore, serine and threonine were studied as these α-amino alcohols are the periodate-oxidizable moieties in adrenocorticotropic hormone (ACTH) and luteotropic hormone (LTH), respectively.

Solution Studies

The *in vitro* periodic acid-Schiff spot test of McManus and Hoch-Ligeti (16) was modified slightly: chilled (4° C) 0.5 per cent periodic acid (H_5IO_6), 0.75 cc, was added directly to dry samples of the test substances. Five minutes after introducing the periodic acid, 3 cc of chilled Schiff reagent (Lillie's "cold Schiff reagent," (14) containing 1 per cent basic fuchsin, C. I. 677, G. T. Gurr, London and 1.9 per cent sodium metabisulfite in 0.15 M hydrochloric acid) was added, the solutions decanted into Beckman 1 cm cuvettes, and extinction readings begun in a DU spectrophotometer.

The spectral absorption, from 425 to 650 mμ, of various concentrations of 4 pituitary tropins, 3 hexoses, and 2 α-amino alcohols were determined. Readings were begun immediately after adding the Schiff reagent, and repeated at 15 minutes and 1 hour. Cuvette covers were used to prevent the loss of SO_2. The entire procedure was carried out at room temperature (20°C). Two blanks were used for each substance tested: the direct Schiff reaction was assessed by a non-oxidized control in which the test material was treated with 0.75 cc distilled water in lieu of the periodic acid; the interaction of the periodic acid with the Schiff reagent was ruled out by a blank cuvette in which these materials were mixed, in the usual proportions.

Stoichiometry

Measurements of extinction values at the absorption maximum (E_{max}) were made at 15 minutes and 1 hour after adding the Schiff reagent. The different concentrations of hormones and standard substances were chosen on the basis of the PAS positivity of the test material in prior runs.

Tissue Studies

Human pituitary glands were fixed in neutral formalin, rinsed thoroughly, and embedded in paraffin; the blocks were sectioned at 5 μ. Pituitaries were obtained fresh from surgical removals. Sections were oxidized for 5 minutes in 0.5 per cent aqueous periodic acid and immersed in the Schiff reagent for

20 minutes. After the customary bisulfite and tap water rinses, they were dehydrated and mounted.

Microspectrophotometry

The microspectrophotometer has been described earlier (4, 23). In the present investigation continuous absorption spectra between 620 and 400 mμ were recorded with a scanning time of 2 minutes. The light source was a tungsten filament lamp and the optics used was the Zeiss Ultrafluor with numerical aperture 1.00. The half-band width was 1.5 mμ and the light absorption values were recorded directly as J_1/J_0 per cent with an accuracy of $\frac{1}{10}$ of a per cent. For measurements, optical cross-section areas in the 5 μ thick sections were selected, 1.5 μ in diameter, from representative parts of Schiff-positive cells or colloid. In some instances, staining with PAS-orange G was done on adjacent, serially cut sections for cytological examination of the structure under measurement.

RESULTS

On adding Schiff reagent to the periodate-treated materials the solutions developed color at variable rates. The direct Schiff reaction of all substances tested, and the periodate blanks, were negative.

Absorption Spectra in Vitro

The development of a characteristic absorption spectrum is presented, for FSH, in Fig. 1. Readings were begun as quickly after adding the Schiff reagent (time = 0) as mixing, decanting into cuvettes, and placing in the Beckman would allow. The spectral characteristics of the developing color were defined by this time. Fig. 1 also demonstrates that the color developed at the 15-minute reading is close to that achieved by 1 hour; when followed for periods up to 3 hours, shape, magnitude, and absorption maximum of the glycoprotein hormones were virtually unchanged.

While all of the substances demonstrated a peak around 560 mμ, the general shape of the absorption curve varied slightly from one substance to another and varied also with time for the same substance. In the case of FSH and mannose (Figs. 1 and 2) the absorption between 510 and 530 mμ increased more with time relative to the maximum absorption at 560 mμ. The final absorption curves of the glycoprotein hormones were practically identical, while those for mannose showed an additional absorption in the short wavelength range.

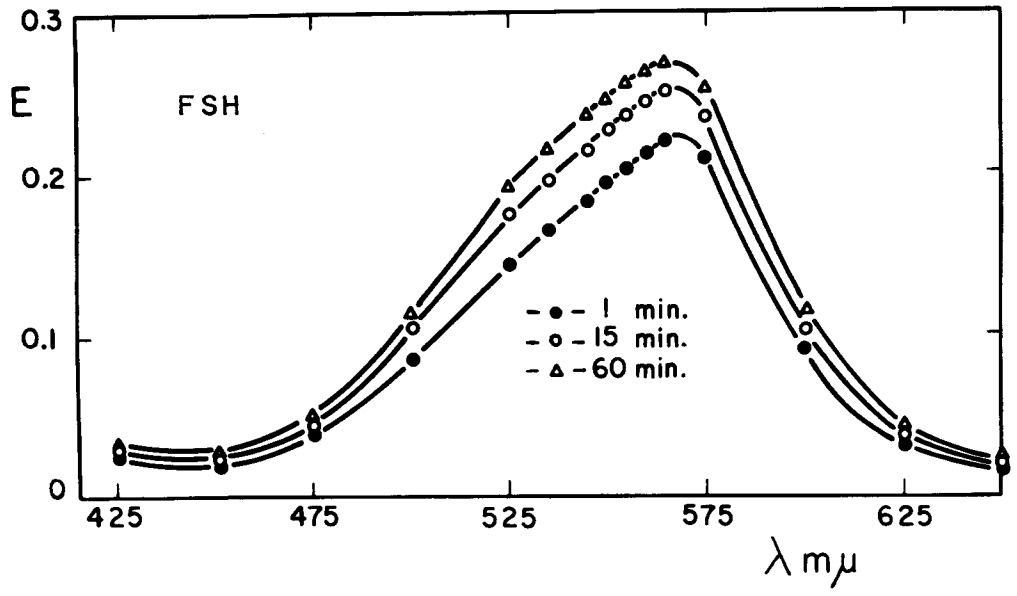

FIGURE 1

Absorption curves for FSH (1 mg) at intervals after adding Schiff's reagent to periodate-treated hormone.

All of the hexoses demonstrated a slower increase in the absorption of the colored Schiff-aldehyde product compared with the glycoprotein hormones, as may be seen in the curves for mannose (Fig. 2).

Fig. 3 depicts variations in the absorption spectrum with time for threonine: the absorption with this substance faded rapidly from the maximum recorded at 1 minute. The absorption spectrum for serine, on the other hand, did not exhibit rapid fading.

Stoichiometry

Table II presents the extinction values at E_{max} for 4 concentrations of the hormones listed. Each entry is the average of 2 replications; the variation due to weighing out milligram amounts was eliminated by taking equal aliquots after dissolving 2 times the amount to be measured in the periodate solution. As Table II shows, there exists a straight-line relationship between concentration and extinction under the present experimental conditions, *i.e.*, the developed color conforms to Beer's law.

Table III compares the extinction values for 1 mg amounts of the 4 hormones. It can be seen that the color development after periodate oxidation is greatest for the gonadotropic hormones, less for TSH, and least for ACTH. In the second column these extinction values are restated in terms of the amount of color generated per unit of biological activity on bio-assay (from data presented in Table I). The color produced by 1 unit of FSH is equivalent to approximately 2 of TSH, 4 of LH, and 30 of ACTH.

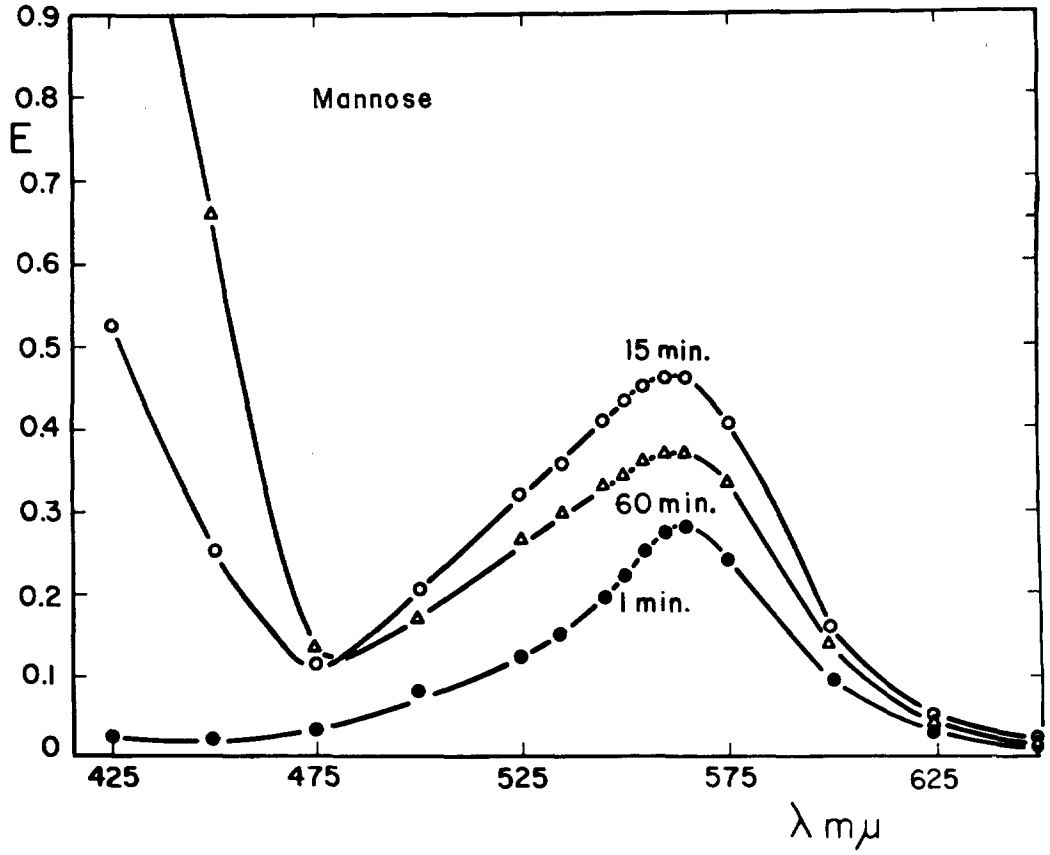

FIGURE 2

Absorption curves for mannose (2 mg) at intervals after adding Schiff's reagent to periodate-treated substance.

TABLE II

Chromogenicity of Hormones in the in Vitro PAS Test

Hormone	Extinction values at absorption maximum*			
	0.5 mg/3.75 cc	1.0 mg/3.75 cc	2.0 mg/3.75 cc	4.0 mg/3.75 cc
FSH	0.094 (±0.003)	0.200 (±0.003)	0.430 (±0.010)	0.964 (±0.044)
TSH	0.062 (±0.001)	0.107 (±0.004)	0.202 (±0.005)	0.428 (±0.011)
LH	0.092 (±0.003)	0.222 (±0.005)	0.428 (±0.010)	1.14 (±0.05)
ACTH	0.014 (±0.001)	0.034 (±0.003)	0.068 (±0.005)	—

* Numbers in parentheses are deviations from tabulated readings.

Microspectrophotometry

Fig. 4 depicts an example of areas measured in a 5 μ section of a normal pituitary. The microspectrophotometer recordings were available as per cent absorption and were converted to the extinction values in the graphs of Fig. 5. The two curves labeled "basophils" are from PAS-positive cytoplasm of well granulated, round, anterior lobe cells; that labeled "colloid" is from PAS-positive colloid. The general shape of the curves conforms

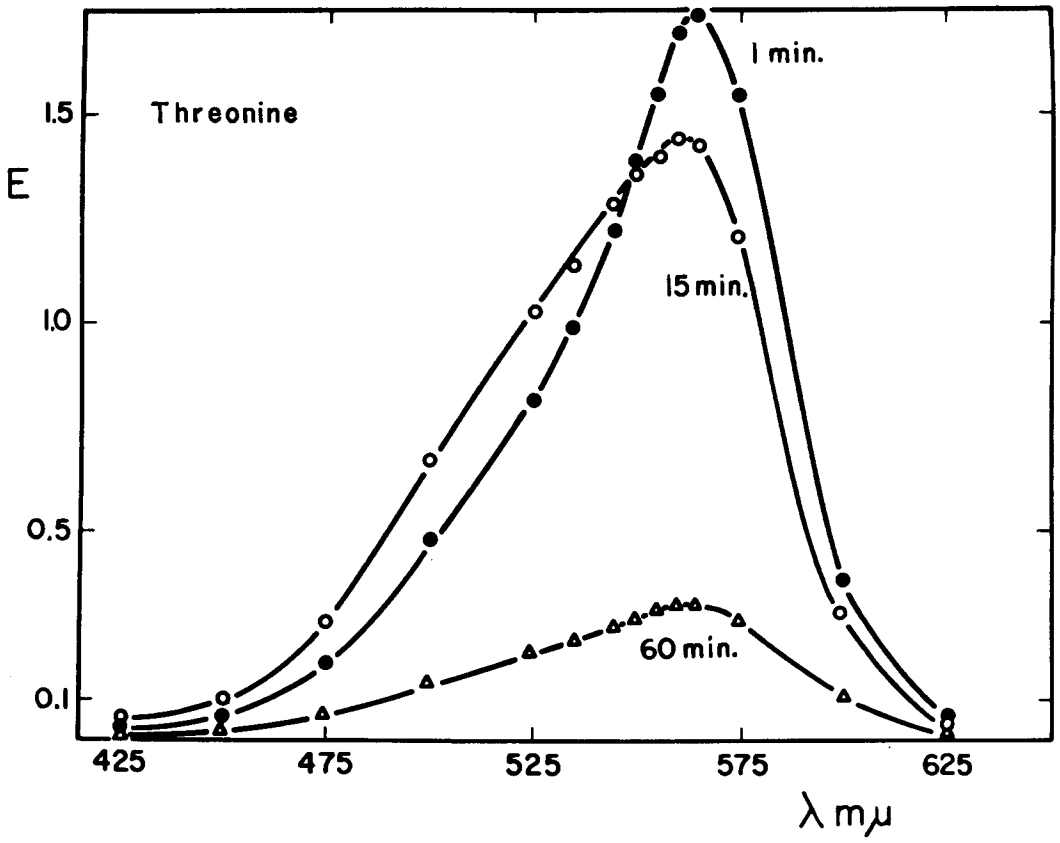

FIGURE 3

Absorption curves for threonine (4 mg) at intervals after adding Schiff's reagent to periodate-treated substance.

to that given by the glycoprotein hormones *in vitro*. Measurements of PAS-positive cells in the posterior lobe yielded spectra similar to those plotted, as did a range of less well granulated anterior lobe basophils.

TABLE III

Comparative Chromogenicity of Four Pituitary Hormones in the in Vitro PAS Test

Hormone	Extinction values at absorption maximum	
	per milligram	per unit of biological activity*
TSH	0.107	0.107
FSH	0.200	0.200
LH	0.222	0.049
ACTH	0.034	0.007

* Numbers in this column are based on bio-assay data presented in Table I.

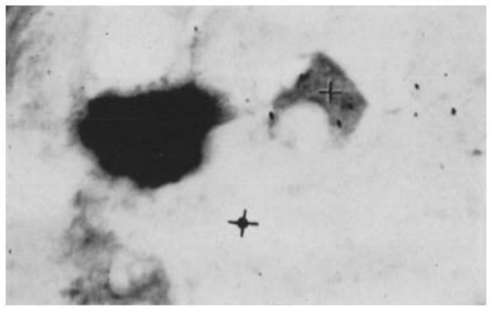

FIGURE 4

PAS reaction in a 5 μ thick section of normal pituitary. Areas of 1.5 μ in diameter in colloid (left) and granulated cytoplasm (right) have been selected for measurements of absorption spectra. The reference area ("blank") is indicated in the space below. Photomicrograph at 560 mμ. Objective aperture 1.0, magnification, approximately 1000.

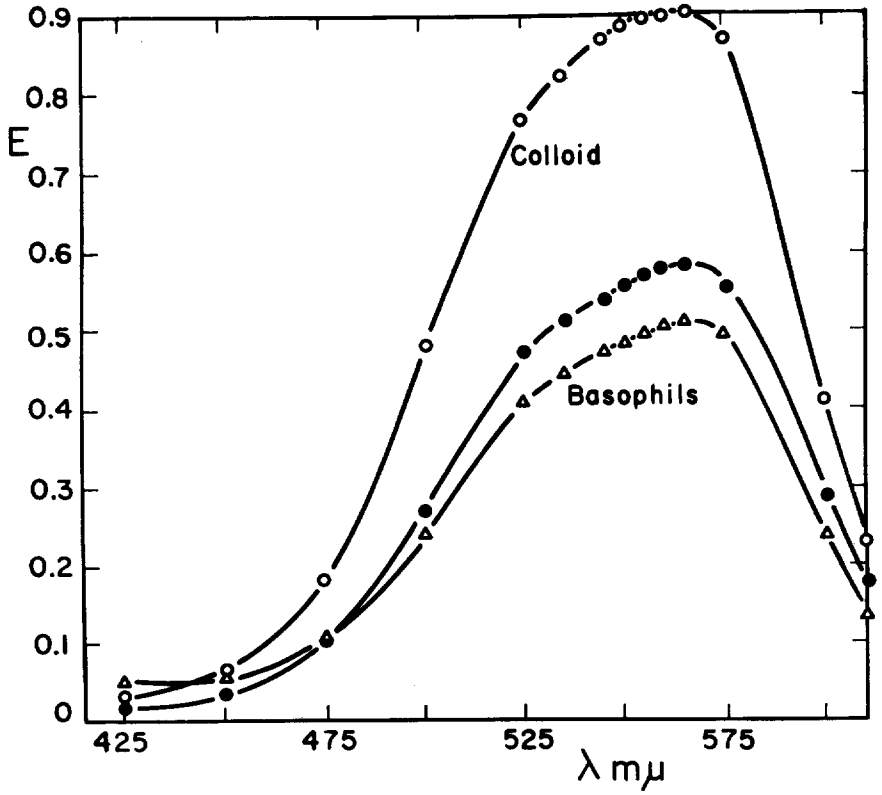

FIGURE 5

Absorption curves of PAS-positive pituitary structures *in situ*.

DISCUSSION

The positive periodic acid–Schiff reaction of the 3 glycoprotein tropins is confirmed in these *in vitro* studies. The absorption maximum and the general shapes of the spectral absorption curves correspond to those obtained in earlier works with Schiff reagent–formaldehyde mixtures (2, 9). Also the rate of color development behaves similarly.

Within the ranges of concentrations studied, the color reaction conforms to Beer's law. In principle, therefore, possibilities exist for estimating the content of glycoprotein hormones in the pituitary histological structures by microspectrophotometry.

Histochemical studies on the nature of PAS-positive substances (11) suggest that, in the absence of glycogen, the only substances in tissue sections which contribute significantly[1] are characterized by hexose and/or methylpentose residues containing free 1,2 glycol groups—the carbohydrate-protein complexes. FSH, LH, and TSH, the glycoprotein tropins, satisfy this requirement. The results in Table III suggest that the type of hexose, or hexoses, in the hormone may influence the yield of aldehyde. Thus the FSH, containing somewhat less than 2 per cent carbohydrate (7), is distinctly more PAS-positive than TSH for which a hexose

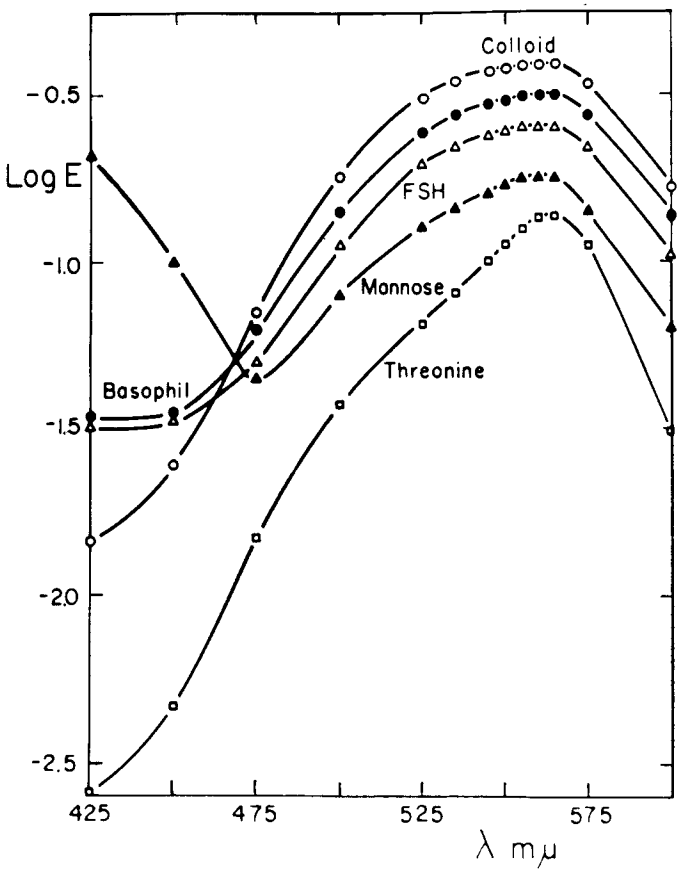

FIGURE 6

Log extinction curves comparing microspectra of pituitary structures with *in vitro* reactions of some PAS-positive test substances.

[1] Pearse (19) points up the possibility of a PAS positive reaction in paraffin sections due to glycolipids and sphingolipids. Although he notes that the former are seldom encountered in normal tissue and the latter have not been demonstrated under the usual conditions of oxidation employed, we plan studies to rule out these interferences.

content of 5.3 per cent was reported by Leblond *al al.* (11); these same workers found both mannose and galactose in TSH while Otsuka (17) cites only mannose in FSH. The influence of the steric position of the hydroxyl groups and the presence and location of other moieties of the molecule is also known to affect the rate of oxidation of reactive groups and thus the yield of aldehyde (5). Caution is required in interpreting the PAS staining of ACTH *in vitro* owing to the purity of the hormone preparation used (20 per cent; see Table I). However, when the color developed is expressed in terms of biological activity (Table III), it appears that the oxidizable α-amino groups in this hormone confer only slight chromogenic properties. This fact may be useful in the interpretation of cytoplasmic staining; for example, the "pale" PAS reaction of ACTH would be inconsistent with strongly Schiff-positive granules.

Fig. 6 shows the log extinction microspectrophotometer curves for the Schiff-positive pituitary structures together with similarly treated spectra from the *in vitro* experiments. The agreement is close between the microspectra and the color developed by purified FSH. The slight deviation at 425 to 450 mμ can be expected from the effect of a non-specific light-scattering component in the histological structures. This general agreement indicates that proportionality between the 6 to 8 different absorbing components (2, 8) of the Schiff-aldehyde reaction is the same in the histological structures as *in vitro*. The PAS reactions of mannose and threonine show, on the other hand, definite deviations.

Thus the microspectrophotometrically obtained data, under the proper experimental conditions and when interferences are excluded, will give a quantitative measure of aldehyde groups generated from the glycoprotein tropins by periodate oxidation in a particular cell structure. Further work is needed to achieve the measurement of a single hormone in localized pituitary structures, for instance, the use of other oxidizing agents and of methods for end-group blockage which may selectively affect one or another of the tropins.

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