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QUATERNARY NITROGEN BASES IN HUMAN THYMUS GLANDS

BY

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Although it is widely recognized that removal of the thymus gland is often of considerable benefit to patients with myasthenia gravis, the manner by which this effect is produced is a matter of conjecture. The problem has been explored in several ways, and these have been reviewed by Wilson and Wilson (1955). Extracts of thymus glands removed from patients with myasthenia gravis were reported to depress the response of the isolated rat diaphragm to stimulation of the phrenic nerve (Wilson, Obrist, and Wilson, 1953), and subsequently Nowell, Wilson, and Wilson (1959) showed that similar effects were produced on the cat tibialis/sciatic-nerve preparation by extracts of foetal whale thymus glands. These effects were not produced by similar extracts prepared from human lymph nodes and human voluntary muscle. The results of further work on the purification of extracts of foetal whale thymus glands by treatment with ion-exchange resin and paper chromatography were reported by Nowell and Wilson (1961), who identified in the extracts two quaternary nitrogen compounds with characteristic R_f values. The presence of two similar compounds was also detected in extracts of thymus glands removed from two patients with myasthenia gravis. These observations prompted a further investigation of the quaternary nitrogen bases in human thymus glands.

Methods

Collection of Thymus Glands

Thymus glands from patients with myasthenia gravis were obtained immediately after thymectomy. Adventitious

tissue was removed, and each gland was usually weighed before and after the excision of a specimen for histological examination. Each gland was placed in an airtight jar containing acetone A.R. and stored in the refrigerator. One thymus gland was also obtained from a patient with myasthenia gravis at post-mortem examination and stored in a similar way.

Normal thymus glands were obtained from neonatal infants and adults at necropsy and similarly stored in acetone. The term "normal" is not easy to define in this context, for many factors may influence the size and the histological structure of the thymus gland. For the present purposes it was thought desirable to exclude thymus glands from patients who had been given corticosteroids or A.C.T.H., or who had died from infectious or cachectic diseases. In one case a thymus gland was obtained from a female patient during operation for coarctation of the aorta.

Method of Extraction

Each thymus gland was extracted with acetone by the method previously described by Nowell *et al.* (1959). The extracts were further processed by passage through a Seitz centrifugal filter with an ultrafiltration membrane and centrifuged at 1,500 r.p.m. for 15 minutes. The filtrate was then passed through a 5.4 by 0.5 cm. "amberlite I.R.C. 50" (100-200 mesh) ion-exchange column and buffered to pH 6.86 with mixed phosphate buffer. After washing with distilled water the column was eluted with 0.2 M

dipotassium hydrogen phosphate; the eluate was collected in six fractions, each of 1.5–2 ml.

Method of Assay

The quantitative estimation of quaternary nitrogen compounds (Q.N.C.) depends on the formation of a chloroform-soluble complex with bromophenol blue. The intensity of blue colour in the chloroform layer is a measure of the concentration of Q.N.C. The standard used for the assay was the quaternary nitrogen base neostigmine, which was arbitrarily chosen because of its stability and sensitivity.

A modification of the colorimetric method of Nowell, Scott, and Wilson (1962) was used. One millilitre of 10% isobutyl alcohol in chloroform was added to 0.3 g. of sodium carbonate in a series of 10-ml. centrifuge tubes. Then 0.5 ml. of bromophenol blue reagent and 0.5 ml. of the standard or test solution were added. After standing for five minutes the tubes were shaken mechanically in a vertical shaker designed to give 2,000 shakes per minute. Five minutes later the tubes were centrifuged at 2,000 r.p.m. for 15 seconds. The tubes were allowed to settle for five minutes and the upper aqueous layer was then aspirated and discarded. The lower blue chloroform layer was transferred to a 2-cm. spectrophotometer microcell and its optical density measured at 605 m μ by a Unicam SP 500 spectrophotometer. The concentration of Q.N.C. was expressed in terms of neostigmine by reference to a calibration curve obtained with different concentrations of neostigmine methyl sulphate in 0.2 M dipotassium hydrogen phosphate.

For comparative purposes it was necessary to express the concentration of Q.N.C. in terms of a standard weight of thymus gland. For this reason all the results are described as the amount in 1 g. of acetone dried thymus gland. This was considered to be a more accurate estimate than the amount in fresh thymus tissue, the water content of which varied; 1 g. of acetone dried powder was usually obtained from 5 to 7 g. of fresh thymus gland.

Two important points of detail concern the use of purified isobutyl alcohol and the necessity to wash all glassware, including the spectrophotometer microcells used for the assay, in chromic-acid cleaning solution. Isobutyl alcohol (A.R.) was purified by washing first with one-fifth vol. N/1 NaOH, then with one-fifth vol. N/1 HCl, and finally two washes with one-fifth vol. distilled water. Ten per cent. purified isobutyl alcohol in chloroform was dried by shaking with anhydrous sodium sulphate (6 g./100 ml.).

Paper Chromatography

Paper chromatography was used to dissociate the bromophenol blue complexes and to establish the R_f values of the Q.N.C. which formed the colour complex; details of this procedure have been published by Nowell and Wilson (1961) and Nowell *et al.* (1962). The blue colour complex obtained by the assay procedure was evaporated to dryness in a distillation flask at 37° C. under reduced pressure. The residue was dissolved in 0.5 ml. of methanol and delivered at a constant rate to the paper by a micrometer syringe. The conditions of chromatography were as follows: solvent, 32 *n*-butanol: 8 ethanol: 12 distilled water: 1 glacial acetic acid; direction, descending; paper, Whatman 541; temperature, approximately 20° C; time, 8 hours; length of run, 40 cm.; paper-drying, over a current of air for 24 hours; developer, iodine vapour (Brante, 1949) brown spots; potassium iodoplatinate solution (Munier and Macheboeuf, 1949) blue spots. The reference substance used was a solution of choline chloride in methanol (10 mg./ml.).

B

Paper chromatography of the blue colour complex was dependent on the amount of complex formed in the course of each assay. In general the equivalent of 30–60 μ g. of neostigmine was applied to the paper.

Results

Myasthenic Thymus Glands

The results of investigating the thymus glands of 15 patients with myasthenia gravis are shown in Table I. With one exception the thymus glands were obtained from patients who had been referred for thymectomy. Macroscopic and histological examination of a specimen of each gland confirmed that all the glands were non-neoplastic.

TABLE I.—Concentration of Quaternary Nitrogen Compounds (Q.N.C.) in Non-neoplastic Thymus Glands Removed from Patients with Myasthenia Gravis

Case No.	Age at Operation (Years)	Concentration of Q.N.C. (μ g. g.)	Duration of Disease (Years)
1	7	79.7	4 1/2
2	11	37.6	9 1/2
3	25	88.7	9 1/2
4	19	19.3	1
5	35	27.0	1
6	24	155.3	1 1/2
7	22	31.5	1 2/2
8	55	40.7	1 3/2
9	12	47.4	1 6/2
10	47	59.4	2 5/2
11	30	15.8	2 6/2
12	22	93.2	3 10/2
13	32	163.3	5
14	19	24.4	10
15	34	34.0	13
Mean		61.2 μ g./g.	
Standard deviation		46.9 "	
Coefficient of variation		76.6%	

It will be seen from Table I that the concentration of Q.N.C. in the glands ranged from 15.8 to 163.3 μ g./g., with a mean value of 61.2 ± 46.9 μ g./g. Although the four highest concentrations were found in thymus glands removed from patients between the ages of 22 and 32, statistical analysis showed no relationship between the age of the patient and the concentration of Q.N.C. in the thymus gland ($r = -0.026$, $P > 0.05$). There is good evidence that one of the most important factors which seem to influence the therapeutic response to thymectomy is the duration of the disease before operation (Keynes, 1946, 1949; Ross, 1952, 1961; Simpson, 1958). In Table I the results are arranged in order of increasing duration of disease, and it will be seen that there is no relation between the Q.N.C. content of the thymus glands and the interval between onset of the disease and operation. This suggests that any beneficial effect from thymectomy is not likely to be determined by the amount of Q.N.C. stored in the thymus gland. This point is discussed in more detail later.

Paper chromatography of the blue colour complex yielded four distinct spots (see Chart); they were clearly separated from the bromophenol blue, which extended from R_f approximately 0.6 to the solvent front. These results are set out in Table II. One of the spots (R_f 0.22–0.27) was identical with the spot obtained by the application of choline chloride to the paper, and was detected in all but three of the thymus glands. Another spot (R_f 0.14–0.22), arbitrarily labelled B3, was identified in all but one of the thymus glands. The chemical identity of the substance giving rise to this spot has not yet been established despite the fact that many attempts were made to match it against a large variety of standard chemical compounds. It will also be seen from the Chart and Table II that two other spots were identified, either alone or together, in 10 of the thymus glands. One of these spots (R_f 0.33–0.36) was labelled B1 while the other, B2, had an R_f value of 0.42–0.44. These two spots were first identified by Nowell and

Wilson (1961) in the bromophenol blue complexes of foetal whale thymus gland extracts. Although there is good evidence that they are obtained from Q.N.C., their exact chemical nature has not yet been established.

Paper chromatography of bromophenol blue complexes of (a) choline, (b) myasthenic thymus glands, and (c) neostigmine methyl sulphate.

TABLE II.—Chromatographic Separation of Q.N.C. from Non-neoplastic Thymus Glands of Patients with Myasthenia Gravis

Case No.	Sex	Age at Operation (Years)	Chromatography				Concentration of Q.N.C. ($\mu\text{g./g.}$)	Duration of Disease (Years)
			Choline	B3	B1	B2		
4	F	19	+	+	+	+	19.3	1
5	F	35	+	+	+	+	27.0	1
7	F	22	+	+	+	+	31.5	1 2/12
8	F	55	+	+	+	+	40.7	1 3/12
9	F	12	+	+	+	+	47.4	1 6/12
10	M	47	+	+	+	+	59.4	2 5/12
13	F	32	+	+	+	+	163.3	5
1	F	7	+	+	+	+	79.7	4 1/2
12	M	22	+	+	+	+	93.2	3 10/12
15	M	34	+	+	+	+	34.0	13
6	F	24	+	+	+	+	155.3	1 1/12
11	F	30	+	+	+	+	15.8	2 6/12
14	F	19	+	+	+	+	24.4	10
2	F	11	+	+	+	+	37.6	9/12
3	F	25	+	+	+	+	88.7	9/12

R_f values: choline, 0.22–0.27; B3, 0.14–0.22; B1, 0.33–0.36; B2, 0.42–0.44.

Further inspection of Table II shows that of the 10 thymus glands which contained B1 and B2 spots either alone or together, 9 were removed from patients who had had the disease for five years or less. This might suggest that there is some connexion between the duration of the disease and the occurrence of these spots in the thymus glands; however, this is unlikely, since four of the patients whose thymus glands contained no B1 or B2 spots had had symptoms for less than three years.

An opportunity was also taken to investigate the thymus glands of six patients with myasthenia gravis where histological examination had shown unequivocal evidence of a tumour. Here the concentration of Q.N.C. in the individual glands ranged from 24.6 to 172.1 $\mu\text{g./g.}$, with a mean value of $78.8 \pm 55.6 \mu\text{g./g.}$ There was no significant difference between the mean value of these glands and those of the non-neoplastic glands ($t=0.75$, $P>0.05$). Although chromatography of the blue colour complexes showed evidence of the B3 spot in all cases, the choline spot was present in only two. A surprising and notable feature was the failure to detect B1 and B2 spots in any of these glands. There is not sufficient evidence to show whether these differences might in part be due to the effects of radiotherapy prior to thymectomy.

Normal Adult Thymus Glands

Thymus glands from 11 subjects were used in this part of the investigation, the results of which are shown in

Table III. Three of the glands (Cases 1–3) were obtained from subjects aged under 21 years. The concentration of Q.N.C. ranged from 0 to 71.1 $\mu\text{g./g.}$, with a mean value of $21.8 \pm 23.2 \mu\text{g./g.}$ Although there is no clear evidence of any relation between age and thymus content of Q.N.C. it is notable that the three glands obtained from subjects over 47 years contained no Q.N.C. The reason for this is obscure.

TABLE III.—Concentration and Chromatographic Separation of Q.N.C. from Thymus Glands of Non-myasthenic Patients

Case No.	Age (Years)	Sex	Chromatography				Concentration of Q.N.C. ($\mu\text{g./g.}$)
			Choline	B3	B1	B2	
1	6	M	+	+			15.1
2	9	M	+	+			12.8
3	15	M	+	+			32.4
4	21	F	+	+			56.8
5	30	M	+	+			71.1
6	32	M	+	+			15.2
7	44	M	+	+			21.3
8	47	F	+	+			15.4
9	48	F					0
10	51	F					0
11	72	M					0
Mean							21.8 $\mu\text{g./g.}$
Standard deviation							23.2 "
Coefficient of variation							106.4%

Chromatograms of the blue colour complexes of each gland yielded two spots in every case. Their R_f values were identical with those of choline and of spot B3. In no case was spot B1 or B2 identified, and in this respect the results obtained from the adult thymus glands differ from those described for the non-neoplastic myasthenic thymus glands.

Neonatal Thymus Glands

The results obtained from 12 thymus glands of infants aged 1–5 days are shown in Table IV. It will be seen that the concentration of Q.N.C. ranged from 14.9 to 80.2 $\mu\text{g./g.}$, with a mean value $36.9 \pm 20.9 \mu\text{g./g.}$ There was no relation between age and thymus content of Q.N.C.

TABLE IV.—Concentration and Chromatographic Separation of Q.N.C. from Thymus Glands of Neonatal Infants

Case No.	Age (Days)	Sex	Chromatography				Concentration of Q.N.C. ($\mu\text{g./g.}$)
			Choline	B3	B1	B2	
1	1	M	+	+			14.9
2	1	M	+	+			45.4
3	2	M	+	+	+	+	33.7
4	2	M	+	+			38.1
5	2	F	+	+	+	+	35.4
6	2	F	+	+			80.2
7	3	M	+	+			17.4
8	3	F	+	+			32.2
9	3	F	+	+			73.9
10	4	M	+	+			25.7
11	4	F	+	+			17.4
12	5	M	+	+			28.1
Mean							36.9 $\mu\text{g./g.}$
Standard deviation							20.9 "
Coefficient of variation							56.6%

Paper chromatography of the blue colour complex yielded in all cases two distinct spots. They were identical with the two spots previously described for choline and B3. It will also be seen from Table IV that in two cases two additional spots were identified as B1 and B2 and in this respect resemble the non-neoplastic myasthenic glands.

Discussion

The most significant feature of these results is the evidence from paper chromatography that the two spots B1 and B2 were identified in more than half the non-neoplastic thymus glands of patients with myasthenia gravis. A crucial point remains to be established—namely, the chemical identity of the substance giving rise to B1 and B2. These results are in general agreement with those reported

by Nowell and Wilson (1961), in so far as they confirm the presence of B1 and B2 spots in myasthenic thymus glands. It is also noteworthy that these authors detected B1 and B2 spots in foetal whale thymus glands but not in adult whale voluntary muscle, human lymph nodes, and human voluntary muscle; in the present study these spots were identified in two of the neonatal thymus glands but in none of the adult normal thymus glands. This evidence, and also the fact that choline and B3 spots were detected in nearly all of the thymus glands studied, raises immediately the question of the validity of the quantitative results.

Although the chemical structure of the compounds has not been established there are good reasons for regarding them as Q.N.C. because of the methods used in their separation. In particular the bromophenol blue reaction has not been described for substances other than Q.N.C. (Auerbach, 1943; Mitchell and Clark, 1952; Friedman, 1958), and much of the work in this laboratory has confirmed this. Since the substances giving rise to these four spots participate in the reaction with bromophenol blue, the quantitative results can be regarded merely as a measure of the total content of Q.N.C., and a more precise method of quantitative analysis is required which will discriminate between the amounts of B1 and B2 and those of B3 and choline.

There is some relation between the present results and those reported by Wilson *et al.* (1953), who showed that extracts of thymus glands from patients with myasthenia gravis caused a depression of the muscle twitch of the rat phrenic-nerve/diaphragm preparation. This effect was also observed with extracts of neonatal thymus glands, but not with similar extracts of thymus glands from normal adults. Furthermore, the results of Nowell and Wilson (1961) have some relevance; they reported that extracts of foetal whale thymus glands which depressed the muscle twitch of the cat tibialis preparation yielded a bromophenol blue complex from which B1 and B2 spots were isolated.

In the present study there were 10 thymus glands that contained B1 and B2 spots, but the presence or absence of these spots did not appear to be related to the duration of the disease, nor to the severity of symptoms before operation as assessed by the neostigmine requirements of each patient. Of the six patients with a thymoma, the one who derived the greatest benefit from thymectomy had the highest content of Q.N.C. in the thymus gland, and had not received radiotherapy before operation. From the small number of thymus glands in this group it is not possible to make any conclusions about the effects of radiotherapy on the content or nature of the Q.N.C., but it is noteworthy that none of these glands contained either B1 or B2 spots.

As has already been mentioned, many attempts were made to establish the identity of the compounds giving rise to B1 and B2 spots by both pharmacological and chromatographic techniques. The pharmacological investigation has been restricted because of the limited amount of material available, but the results of such experiments as have been possible show that an extract containing these two isolated bases potentiates the response of the frog rectus muscle to acetylcholine and reverses the effect of tubocurarine.

Comparative studies in which the R_f values of a large number of chemical compounds were compared with those of B1 and B2 gave little indication of the structure of these bases. Recent experiments of this kind, however, suggest that the R_f value for spot B1 is very close to that of triethylcholine and that spot B2 closely corresponds in R_f value with diethylcholine (Nowell and Wilson, 1962). These findings are particularly relevant to the results reported by

Bowman and Rand (1961a, 1961b) that the injection of triethylcholine into conscious animals produced muscular weakness which was more severe when the animals were exercised. These authors, indeed, advanced the hypothesis that triethylcholine may interfere with the synthesis of acetylcholine at the neuromuscular junction, and this is consistent with the concept which had previously been suggested by Desmedt (1958, 1961), that the essential lesion in myasthenia gravis is due to a presynaptic biochemical defect.

It is, of course, feasible that triethylcholine or other ethyl derivatives of choline may be released from the thymus glands of patients with myasthenia gravis, and that this may result in a disturbance of neuromuscular transmission. This is likely to be a complex effect, however, because all the evidence shows that the actions of these compounds are not antagonized by anticholinesterase drugs, whereas a basic feature of the myasthenic syndrome is the dramatic relief of symptoms by neostigmine or edrophonium. The presence of analogues of choline particularly at or near the motor-nerve endings might compete with or replace choline in the mechanisms which give rise there to the formation and release of acetylcholine, and in this way they might indirectly contribute to a presynaptic biochemical defect.

It is well recognized that the signs and symptoms of myasthenia gravis are often transient and may indeed disappear; but sooner or later they become permanent, and this suggests that up to a point the change is reversible but later becomes irreversible. Thus there is good evidence that the beneficial effects of thymectomy are most often seen where the disease is of short duration. In these circumstances removal of the thymus gland before the changes become irreversible would presumably eliminate the interaction of the ethyl analogues of choline at the motor-nerve endings. On the other hand, this operation would have little or no beneficial effect after irreversible changes had taken place in patients who had had myasthenia gravis for many years.

Summary

An investigation is described of the quaternary nitrogen compounds (Q.N.C.) present in extracts of human thymus glands by a colorimetric method which depends on the formation of a chloroform-soluble complex with bromophenol blue. The corresponding complex formed by the quaternary nitrogen compound neostigmine was used as a convenient standard for the assay of Q.N.C. in thymus glands obtained from patients with myasthenia gravis and in post-mortem specimens of neonatal infant and normal adult thymus glands.

The results of the assay have shown that there is considerable variation in the Q.N.C. content within each group of thymus glands and that the mean value of the myasthenic thymus glands is greater than that of the two normal groups. The interpretation of these results, however, is obscure in view of the evidence obtained from paper chromatography of the blue colour complex.

Paper chromatography of the blue colour complex formed in the course of each assay showed that there were significant differences between the three groups of thymus glands in respect to the R_f values of the spots which separated from the bromophenol blue. Four types of spots were each characterized by their R_f value, but only one, choline, was identified. Two of these spots (B1 and B2) were observed in 10 of the 15 myasthenic thymus glands, but in only 2 of the 12 neonatal thymus glands and in none of the 11 adult normal thymus glands.

The significance of these findings is discussed in relation to current work on the nature of myasthenia gravis.

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INCIDENCE OF RENAL LESIONS IN POLYCYTHAEMIA

A SURVEY OF 91 PATIENTS

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[WITH SPECIAL PLATE]

Since Bliss (1929) described the case of a patient with hypernephroma and polycythaemia, which he considered to be an incidental complication, increasing numbers of cases with this association have been reported. Forsell (1946) suggested that there was a causal relationship between the two conditions, and subsequent experience has confirmed this beyond doubt. The incidence of renal carcinoma in patients with polycythaemia has been found to be about 2–4% (Videbaek, 1950; Lawrence, 1955; Damon, Holub, Melicow, and Uson, 1958). The incidence of polycythaemia in patients with renal carcinoma is about 2–3% (Berger and Sinkoff, 1957; Conley, Kowal, and D'Antonio, 1957; Damon *et al.*, 1958; Hewlett, Hoffman, Senhauser, and Battle, 1960; Ways, Huff, Kosmaler, and Young, 1961). Most of these analyses were retrospective and the criteria for the diagnosis of polycythaemia not clearly defined. The figures may therefore not be entirely reliable.

Benign renal lesions have also been described in association with polycythaemia. These include renal adenoma (DeMarsh and Warming, 1955) and cystic lesions—solitary cyst (Nixon, O'Rourke, Rupe, and Korst, 1960), multiple cysts (Nixon *et al.*, 1960; Jones, Payne, Hyde, and Price, 1960; Cohen, 1960; Ways *et al.*, 1961; Delamore, MacDonald, and Samuel, 1962), and polycystic disease (Kurrle, 1954; Forsell, 1958; Nixon *et al.*, 1960; Friend, Hoskins, and Kirkin, 1961). Hydronephrosis has been reported with polycythaemia; it may be unilateral (Cooper and Tuttle, 1957; Gardner and Freymann, 1958; Castleman and Kibbee, 1959; Lawrence and Donald, 1959; Jones *et al.*, 1960; Ellis, 1961; Ensor, 1961; and Martt, Sayman, and Neal, 1961), or bilateral (Jaworski and Hirte, 1961).

Other tumours as well as renal ones have been found in association with polycythaemia. These include brain

tumours, especially cerebellar haemangioblastoma (Brody and Rodriguez, 1961), fibromyomata of the uterus (Singmaster, 1957), hepatocellular carcinoma (Kan, McFadzean, Todd, and Tso, 1961), hamartoma of the liver (Josephs, Robbins, and Levine, 1962), pheochromocytoma (Bradley, Young, and Lentz, 1961), and tumours causing Cushing's syndrome (Videbaek, 1950).

That the relationship between the renal lesion and the polycythaemia has been causal and not merely coincidental has been proved on many occasions by the surgical removal of the lesion being followed by remission of the polycythaemia. For instance, prolonged remission for 11 years after removal of a renal carcinoma without any other treatment of the polycythaemia has been reported (Damon *et al.*, 1958), as well as many remissions of shorter duration (Conley *et al.*, 1957; Campbell, Pasquier, St. Martin, and Worley, 1958; Damon *et al.*, 1958; Frey, 1958; Ewert, 1960; Barnard, 1961; Ensor, 1961; Ways *et al.*, 1961). An exceptionally significant case was reported by Omland (1959). The polycythaemia remitted after nephrectomy for carcinoma. Relapse was associated with the development of metastases on two occasions in the next nine years. On each occasion remission of the polycythaemia followed irradiation of the metastases.

Benign renal lesions associated with polycythaemia have also been treated surgically. Thus remissions have been reported after nephrectomy or partial nephrectomy for renal cysts (Kurrle, 1954; Forsell, 1958; Jones *et al.*, 1960; Nixon *et al.*, 1960) and after nephrectomy for hydronephrosis (Cooper and Tuttle, 1957; Gardner and Freymann, 1958; Lawrence and Donald, 1959; Jones *et al.*, 1960; Ellis, 1961; Ensor, 1961; Martt *et al.*, 1961).

It is now generally accepted that the kidney produces a factor, "erythropoietin," which stimulates erythropoiesis. Recently, assays in patients with renal lesions and poly-