Experience with a cholinesterase histochemical technique for rectal suction biopsies in the diagnosis of Hirschsprung’s disease


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SYNOPSIS  Cryostat sections from 160 rectal suction biopsies were stained for cholinesterases by the method of Karnovsky and Roots (1964) in an attempt to facilitate the diagnosis of Hirschsprung’s disease.

The method proved at least as reliable as experienced assessment of paraffin haematoxylin-eosin sections, and appeared to offer the advantages of reduced scanning fatigue and superior demonstration of the increased cholinesterase-positive nerves in Hirschsprung’s disease.

Contrary to the findings of Meier-Ruge (1971) it was not possible to base a diagnosis on mucosal cholinesterase activity.

Rectal suction biopsies taken by the simple technique recently described by Noblett (1969) and Campbell and Noblett (1969) are surgically preferable to larger submucosal biopsies or to full-thickness techniques.

To the histopathologist, however, they may present problems of interpretation in the diagnosis of Hirschsprung’s disease. This is in part due to the relatively diffuse nature of the superficial submucous plexus compared with the more familiar inter-muscular plexus and to the rather smaller size of its ganglion cells and ganglion complexes.

We have applied a cholinesterase (ChE) histochemical technique to biopsies of this type in an attempt to make the submucosal nervous tissue more conspicuous and also to evaluate the significance of mucosal cholinesterase activity in the diagnosis of Hirschsprung’s disease.

Materials and Methods

The biopsy site was in all cases estimated to be at least 1 cm above the proximal limit of the ‘normal hypoganglionic zone’ described by Aldridge and Campbell (1968).

One hundred and sixty biopsies were taken from 101 patients ranging in age from 2 days to 17 years, and were processed for cholinesterases. Whenever possible duplicate biopsies were also taken from an adjacent site, and were processed by a conventional histological method (fixation in neutral 10% formalin, embedding in paraffin wax, thin serial sections stained with haematoxylin and eosin). All the patients had presented with a clinical picture suggesting the possibility of Hirschsprung’s disease, which was ultimately confirmed by definitive surgery in 33 of them and was excluded in the remainder.

Histochernical Technique

The biopsy was immediately spread submucosa-down onto a small block of animal spleen or liver (for ease of handling and cutting), and quick frozen in hexane cooled by dry ice in acetone. It was then cut at 15μ in a plane perpendicular to the flattened submucosal surface, as serial sections. The slides were air dried for a few minutes, immersed for 30 seconds in 10% neutral formalin at room temperature, and washed in distilled water. They were incubated for three hours at 37°C in the medium described by Karnovsky and Roots (1964), using an acetate buffer at pH 6·0 and s-acetylthiocholine iodide (AThChI) as substrate. Inhibitors were not routinely used, so that the histochemical reaction presumably demonstrated both true (acetyl-) and pseudo-cholinesterase activity. After rinsing in
distilled water the slides were counterstained in Ehrlich's haematoxylin to show the nuclei, differentiated in 1% acid alcohol, blued in running tap water, dehydrated, cleared in xylol and mounted in Canada balsam.

The specificity of the histochemical reaction was assessed by comparing serial sections of representative biopsies treated in different ways, including the use of selective acetylcholinesterase and pseudo-cholinesterase inhibitors. Counterstaining was omitted in these tests.

Results

Appearance of ChE preparations
The end product of the histochemical reaction is a finely granular and highly refractile deposit of red-brown copper ferrocyanide. Localization in our sections was good.

Submucous ganglia
The submucous ganglia appear as strongly ChE-positive bodies of more or less clearly defined outline, though highly variable in size and shape. The ganglion cells themselves are rather less ChE positive, and their loci typically appear as large, pale, approximately spherical spaces sharply differentiated from the surrounding matrix. Smaller and denser nuclei of supporting cells are generally visible within the ganglion (fig 1).

Nerve bundles
Nerve bundles are the only other strongly ChE-positive bodies present throughout the submucosa. In longitudinal section they appear as dense, more or less undulating ('zig-zag') bundles of fibres, in which individual axons are not distinguishable. Numerous elongated, densely basophilic nuclei of supporting cells are seen along their length, especially around their periphery. Even in oblique or cross section their fibrous appearance and lack of ganglion cells distinguishes nerve bundles from ganglia (fig 2).

A loose mesh of fine ChE-positive nerves is also

Fig 1
A typical (fairly large) submucosal ganglion (× 375). Note the characteristic ‘halo’ of paler cytoplasm around each ganglion cell nucleus.

Fig 2
A typical (fairly large) ChE-positive submucosal nerve (from a ganglionic biopsy) (× 150).
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appearance, associated with brown-staining oval cell nuclei, occur in the lamina propria of many biopsies, both ganglionic and Hirschsprung’s. These fibres do not appear to be nerves: in some biopsies they occur throughout the mucosa (with variable breakdown of the normal crypt architecture) and constitute the dominant contribution to ChE activity. They are consistently seen in biopsies which appear congested on macroscopic examination. They probably consist of extravasated plasma proteins and blood cells, and may in some cases be due to the biopsy procedure itself. Similar patches are sometimes also seen in the submucosa.

As well as a brown coloration of the distal cytoplasm of the epithelial cells bordering the lumen, a thin line of ChE-activity is sometimes seen just deep to the basement membrane of the epithelium bordering the lumen. Finally, small branches of the muscularis mucosae penetrate the lamina propria and contribute to the overall activity observed.

ASSESSMENT OF MUCOSAL CHOLINESTERASE ACTIVITY
Assessment of mucosal cholinesterase activity presented several problems. As described above, the pattern as well as the overall average density of ChE coloration varied in several ways. For the purposes of numerical analysis, however, the only practicable criterion was found to be the overall density of the histochemical deposit, regardless of fine histological appearance. The strongly ChE-positive cores of lymph follicles, which were commonly seen in biopsies of both types, were, however, excluded from this assessment.

The biopsies were subjectively ascribed to one of five arbitrary categories thus:

Mucosal activity light
(for the most part barely visible, with at most sporadic patches of higher density) . . rating 1

Mucosal activity moderate
Mostly patchy . . . . . rating 2
Mostly diffuse . . . . . rating 3

Mucosal activity intense
(strikingly increased throughout the greater area of the mucosa)
Mostly patchy . . . . . rating 4
Mostly diffuse . . . . . rating 5
A higher rating figure infers a higher overall average density of coloration.

Numerical Data

ADEQUATE BIOPSIES
Table I shows the distribution of ganglia in biopsies

Fig 3  Muscularis mucosae from a Hirschsprung biopsy (× 190). The superabundant fine nerves are clearly visible against the paler ChE coloration of the smooth muscle, and they are commonly seen even in those Hirschsprung biopsies which do not contain abnormally large or numerous submucosal nerve trunks.

generally visible around the submucosal blood vessels.

Muscularis mucosa
The muscularis mucosae appears as a fairly dense ChE-positive network surrounding the blue-stained elongated muscle-cell nuclei. Examination under high power suggests that the ChE activity is concentrated around the periphery of the muscle cells (fig 3).

Mucosal cholinesterase activity is in most biopsies due in large part to an anastomosing network of fine fibres in the lamina propria, running more or less parallel with the crypts. Their appearance suggests that they are small bundles of nerve fibres. Both their apparent number and their density of histochemical coloration contribute to the overall variability of ChE activity.

In addition, variable patches of intensely ChE-positive material of a more disorganized fibrous
judged adequate in terms of the amount of submucosa of acceptable histological quality available for inspection (as in tables II and III, numbers refer to biopsies and not to patients). The distribution of ChE-positive nerves in the submucosa is also indicated, assessed subjectively as 'few', 'moderate', or 'many', a combined estimate of number and size.

### Inadequate Biopsies

Table II shows the ganglion distribution in biopsies judged inadequate on the above basis and also includes three biopsies in which there was histological evidence that the biopsy site was too low, close to the mucocutaneous junction.

### Mucosal Cholinesterase

Table III shows the mucosal cholinesterase rating of the biopsies against definitive diagnosis. Thirteen biopsies had to be excluded as unsuitable for this assessment.

As an indication of the reproducibility of estimates of mucosal cholinesterase activity for a given patient and height of biopsy, we compared mucosal ratings of pairs of biopsies, each pair taken from one patient within an estimated 1 cm height from each other. In only four instances out of 66 did the pairs differ by more than one rating.

### Specificity Tests

Preincubation of the slides in a solution of N-ethyl maleimide (at 0.1 M in a 0.1 M phosphate buffer, pH 7.4, at 37°C for 15 minutes) resulted in some reduction in nuclear staining, especially noticeable in the mucosa. Karnovsky and Roots (1964) suggest the use of this reagent for abolishing possible reduction of the potassium ferricyanide by sulphydryl groups. The staining reactions of other tissue components were apparently unaltered.

Inclusion of the selective pseudocholinesterase inhibitor iso-OMPA in the incubation medium at $7 \times 10^{-5}$ M resulted in a reduction in staining...
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intensity in the muscularis mucosae and to a lesser extent in the ganglia. Other staining reactions were unaltered.

Inclusion of the selective acetylcholinesterase inhibitor BW 284C51 in the incubation medium at 3 \times 10^{-5} \text{M} resulted in abolition of coloration except in the muscularis mucosae and in the ganglia, in both of which the staining intensity was greatly reduced.

Inclusion of both iso-OMPA and BW 284C51 at these concentrations abolished all but the faintest coloration in the muscularis mucosae.

Discussion

The interpretation of rectal suction biopsies in the diagnosis of Hirschsprung's disease presents several critical questions to the histopathologist. The biopsy site must be selected above the 'normal hypoganglionic zone' described by Aldridge and Campbell (1968), but must be low enough to avoid missing an 'ultrashort segment' variant of the disease. Moreover, since the biopsy is performed without proctoscopy, and on an unsedated child, and in view of the elasticity of the rectal mucosa and the probability of encountering rectal folds, care is necessary to ensure that the surgeon's estimate of biopsy height is reliable. The apparatus must be capable of providing undamaged biopsies of adequate size and depth, and the biopsy must be orientated and processed with special care because of its small size.

Gherardi (1960) and others have established that the limit of aganglionosis in Hirschsprung's disease coincides closely in the submucosal and intermuscular plexuses. Aldridge and Campbell (1968) have significantly advanced our knowledge of the distribution of submucosal ganglia in the normal rectum at different ages, so that the optimum biopsy level for a child of any given age may be estimated with some confidence. Yet many pathologists are still reluctant to make a positive diagnosis of Hirschsprung's disease on the basis of rectal suction biopsies, possibly due both to doubt as to the amount of submucosa that must be scanned before absence of ganglion cells is indicative of aganglionosis at that level; and the relative difficulty and tediousness of accurate identification and assessment of submucosal ganglia and nerves by comparison with the more compact and familiar intermuscular plexus.

Several workers have introduced histochemical methods in an attempt to facilitate the interpretation of these biopsies. Gannon, Burnstock, Noblett, and Campbell (1969) have applied the formaldehyde-induced fluorescence technique for catecholamines developed by Falck and Owman (1965). They were able to diagnose all of eight cases of Hirschsprung's disease studied in this way 'by looking at one, or at most several, sections', on the basis of a massive increase in the fluorescent nerves in the submucosa and muscularis mucosae, compared with normal controls. Though they assert that this method serves as a 'useful corollary to conventional histological methods', the technique is demanding in time and apparatus and is notoriously capricious in its working. Furthermore, Garrett and Howard (1969) found in a similar series at least one apparent exception to these observations.

Garrett and Howard (1969) and Lake (1972) have shown the practicability of rapid non-specific esterase techniques in the demonstration of nerves and ganglia in intraoperative Hirschsprung biopsies. We have applied such a technique to rectal suction biopsies, but found the cholinesterase technique described here more revealing and, apart from the speed, no less convenient.

Meier-Ruge (1968), Moser, Widmer, and Meier-Ruge (1971) and Meier-Ruge, Lutterbeck, Herzog, Morgor, Moser, and Schärli (1972) report on the application of acetylcholinesterase techniques to rectal suction biopsies. Meier-Ruge (1971) and Meier-Ruge, Bielsé, Wiederhold, and Meyerhofer (1971) have routinely used the method of Karnovsky and Roots, as slightly modified by el-Badawi and Schenk (1967), as well as Gomori's (1952) modification of the method of Koelle and Friedenwald. Drawing from experience with a large series of some 214 biopsies from as many patients, he states that a positive diagnosis can be made from rectal suction biopsies consisting of mucosa alone, based on a massive increase in AChE activity in the lamina propria, which he finds to be proportional to the severity of clinical presentation. In normal rectal mucosa, he finds, by contrast, 'virtually imperceptible' mucosal AChE activity (with 90 minutes' incubation). Furthermore he asserts that mucosal AChE activity affords precise diagnosis even 'from biopsies of the anal mucosa and within the first 3 cm of the rectum proximal to the anal ring'.

Garrett, Howard, and Nixon (1969a, 1969b), in 19 cases of Hirschsprung's disease examined, established a positive correlation between the severity of clinical presentation and numbers of AChE-positive nerves in the circular muscle of the distal aganglionic bowel. Of these 19, three cases which failed to show a classic constricted segment on barium enema had even fewer AChE-positive nerves in the circular muscle than did normal controls, and, contrary to Meier-Ruge's findings, also had few in the mucosa. In these three patients presentation was mild (constipation).

We found that from a technical point of view our method presented no special problems, and that
serial sections could with practice be cut on the
cryostat with ease and speed, mounting on average
some 24 sections per slide. Ideally, a biopsy could be
ready for examination within some four hours of
receipt. In the routine pathology laboratory, how-
ever, geared primarily to paraffin sections stained
with haematoxylin and eosin, considerable extra
labour would probably be required to produce
cholinesterase sections solely for rectal suction
biopsies.

The histochemical reaction was generally consist-
tent enough to permit a meaningful subjective
comparison of the ChE activity of different biopsies.
The animal tissue on which the biopsies were
mounted provided a useful control in this respect.
A few biopsies failed to stain properly, ChE-positive
material appearing a dirty green colour: this was
traced to contamination by excess mineral oil used
to lubricate the biopsy forceps. A distinct but slight
increase in the overall histochemical activity was
noted when one sample of AThChI was replaced by
a fresher sample from a different source, and allow-
ance was made for this in the assessment. Cold
storage of the biopsies (protected against drying) or
the air drying of slides at −30°C did not appear to
diminish histochemical activity over a period of a
few days.

The relatively thick sections and protracted incu-
bation period were chosen to demonstrate mucosal
ChE activity to best advantage at the expense of
some loss of cytological detail. Identification of
ganglia was accordingly more dependent on their
overall configuration (though highly distinctive
using this method), rather than on the fine cyto-
logical characterization of ganglion cells, particularly
of the nuclear detail, that fine paraffin sections
permit.

Of the 91 biopsies judged ‘adequate’ (table I)
Hirschsprung’s disease could be excluded by the
presence of ganglia in 47 (52%), and was strongly
suggested in 31 (34%) by the absence of ganglia
coupled with a marked increase in the number
and/or size of ChE-positive nerves in the sub-
mucosa. The remaining 13 (14%) adequate biopsies
were not diagnostic. In no case was the disease
wrongly excluded or confirmed.

By comparison, of the 69 biopsies judged ‘in-
adequate’ from a priori considerations (table II), 24
(35%) excluded Hirschsprung’s disease and 14
(20%) tended to confirm it, leaving 31 (45%)
equivocal. Again no errors of diagnosis were made.

If the rate of diagnosis is considered per patient
rather than per biopsy we find that of 67 patients
from whom an adequate biopsy was obtained, a
correct diagnosis was made for 59 (88%), and
diagnosis was suggested (though less definitely) in a
further five patients (7%).

We found that both ChE-positive nerves and
ganglia in the submucosa could be scanned rapidly
and accurately, using low power (× 100) with only
occasional need for a higher power. The conspicuous-
ness of nervous tissue against the submucosal ground
matter, in our opinion, made scanning of serial
sections for ganglia less tiring than with conven-
tional haematoxylin and eosin preparations. In
particular we felt that the superabundant fine nerves
in and near the muscularis mucosae, which were
demonstrable in nearly all Hirschsprung biopsies,
were easier to assess. But such comparisons are
necessarily subjective, and for the pathologist who
is familiar with the interpretation of paraffin/
haematoxylin and eosin sections of these biopsies,
the advantages may be offset by the problems of
adjustment to a radically different type of prepara-
tion.

A visiting pathologist with special interest and
experience in this field examined a duplicate set of
serial paraffin/haematoxylin and eosin preparations
of biopsies of the last 62 of the series presented here:
his diagnostic rate almost exactly coincided with
our own (Carter, 1972).

Thus, apart from any advantages of convenience,
any ultimate advantage of this method must lie
with the diagnostic potential of the mucosal choline-
terase activity. But though we found (table III) that
most (71%) non-Hirschsprung biopsies had a low
mucosal rating (1 or 2) and most (93%) Hirsch-
sprung biopsies had a higher rating (3, 4, or 5) there
was a considerable overlapping group, so that in
our hands the mucosal cholinesterase activity is not
alone a reliable diagnostic criterion.

In this respect our results differ markedly from
those of Meier-Ruge and his colleagues (1972), who
found high mucosal AChE activity to be a 100% 
reliable criterion for the diagnosis of Hirschsprung’s
disease in a series of 214 patients. This could concei-
vably be due to their routine use of OMPA as a
pseudocholinesterase inhibitor, although our spe-
cificity tests suggest that its use would not have
significantly affected our mucosal observations.
Alternatively the discrepancy may be due to other
differences in technique, including perhaps the
biopsy procedure itself: or differences in inter-
pretation may be involved, our rating being based
solely on the density of histochemical coloration
without interpretation of its origin as nervous,
‘specific’, or otherwise.

Finally it is noteworthy that one biopsy from this
series, taken in the routine fashion from a premature
neonate, contained in addition both circular and
longitudinal muscle (and a ganglionic myenteric
plexus). The biopsy was only of average (absolute) depth but the bowel wall was unusually thin. A similar biopsy has since been taken from a premature baby (not of this series). No complications ensued in either case, but the bowel was presumably nearly perforated, suggesting that a modified technique might be advisable for premature babies. Furthermore, Carter (1972) remarked on the difficulty of identifying ganglion cells in the submucosa of haematoxylin and eosin preparations of biopsies from premature infants. In ChE preparations the ganglia tend to be atypical, with a light histochemical deposit and closely packed ganglion cell nuclei: their identification, however, is unambiguous (fig 4).

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References


Lake, B. D. (1972). Personal communication.


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Fig 4  Submucosal ganglion from a premature infant. (× 450).
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