

4. The relationship of this pathway to the biosynthesis of fungal tetrone acids is discussed.

5. During growth of *A. tenuis* in undisturbed surface culture, the concentration of tenuazonic acid increases rapidly during the second week, then decreases to a minimum and rises again.

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*Biochem. J.* (1961) **78**, 418

## Tissue-Specific and Species-Specific Esterases

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Although electrophoresis has been used extensively to study the protein components of biological fluids, few attempts have been made to investigate soluble proteins from solid tissues by this means, mainly because classical techniques give inadequate resolution of complex mixtures. Zone electrophoresis in starch gels (Smithies, 1955) permits sharper separation and greater resolution, and in the modification introduced by Hunter & Markert (1957) can also be used for specific proteins by applying methods derived from histochemistry. For instance, by applying the method of Nachlas & Seligman (1949) these authors demonstrated several bands with esterase activity in starch-gel-electrophoresis diagrams of aqueous extracts of mouse tissues.

Electrophoresis of serum proteins on starch gels has revealed differences between species (Latner & Zaki, 1957) and even among individuals of the same species (Ashton, 1957; Smithies, 1955, 1959). By other techniques Augustinsson (1958) has demonstrated that there are considerable differences in the serum esterases of different species.

In this investigation starch-gel electrophoresis was used to compare the tissue esterases of a variety of organs in several species, in order to investigate the amount of variation among proteins

with the same enzyme activity. Typical patterns were found for each tissue, and their constancy was demonstrated by their persistence in mammalian cell strains, which had been cultivated *in vitro* for long periods.

#### MATERIALS AND METHODS

*Tissues.* Animals were killed by exsanguination after stunning or after anaesthetizing with ether. The organs were immediately removed and treated as described below. Human tissues were obtained from the post-mortem room, washed thoroughly with 0.15M-NaCl immediately after removal, and frozen at  $-10^{\circ}$  until required. Cell strains were grown as monolayers on glass by standard techniques (Paul, 1959). They were washed *in situ* with 'balanced salt solution' (Hanks & Wallace, 1949) and harvested by scraping from the glass with a rubber 'policeman' before the preparation of extracts. Except for the strains described below, cells were derived from recently disaggregated tissues. Cell strains used were the mouse subcutaneous (fibroblast) strain L, clone 929 (Sanford, Earle & Likely, 1948), the human cervical carcinoma cell, strain HeLa (Gey, Coffman & Kubicek, 1952), the human epithelioma cell, strain HeP 1 (Toolan, 1954) and the human foetal liver (parenchymal) cell, strain HLM (Leslie, Fulton & Sinclair, 1956).

*Preparation of extracts.* The method of preparation of tissue extracts was found to affect the pattern obtained.

Methods tried included simple extraction with water, balanced salt solution and various buffer solutions and also extraction with the same solutions accompanied by ultrasonic treatment for various periods. Extraction with water or balanced salt solution (0.15M) gave identical results, and the electrophoretic patterns obtained were not affected by ultrasonic treatment. However, when extraction was performed with 0.06M-buffers [2-amino-2-hydroxy-methyl-propane-1:3-diol (tris) or phosphate] at pH 7.3 or 8.3, different patterns were obtained (Fig. 1), and ultrasonic treatment led to the progressive disappearance of some bands and increased intensity of others on the electrophoresis pattern. The final procedures adopted were as follows.

*Extracts from solid tissues.* The tissue was first ground with 0.5 vol. of iso-osmotic salt solution or balanced salt solution in a Potter-Elvehjem homogenizer. When a fine uniform homogenate had been obtained it was placed in an ice bath and subjected to ultrasonic treatment for 15-20 min., with a Mullard ultrasonic drill (50 w, 20 kcyc./sec.).

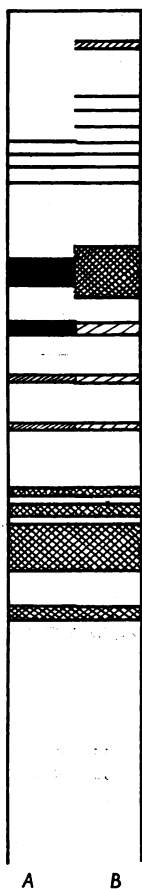


Fig. 1. Comparison of the zymograms obtained from mouse-liver extracts prepared with (A) 0.06M-phosphate buffer and (B) water. Relative intensities of bands are indicated by shading. (The phosphate buffer, pH 8.3, was a mixture of  $\text{Na}_2\text{HPO}_4$  and  $\text{NaH}_2\text{PO}_4$ .)

Finally, it was centrifuged at 35 000 g for 2 hr. at 0°. The clear supernatant was used for electrophoresis.

*Extracts from cultured cells.* Cells were suspended in 0.5 vol. of balanced salt solution and subjected to ultrasonic treatment for 2-3 min. The suspension was centrifuged at 15 000 g for 2 hr. at 0°, and the clear supernatant used.

*Electrophoresis.* The apparatus and general method were essentially those of Smithies (1955). Single-electrode vessels were used at the anode and cathode, and simple carbon electrodes were found adequate. In earlier studies AnalaR soluble starch (British Drug Houses Ltd.) was used for making the gels, batches being tested until a suitable one was found. Starch Hydrolysed (Connaught Laboratories) was used in the later studies.

The discontinuous buffer system of Poulik (1957) was used. The gel was prepared in a buffer, pH 8.64, composed of 0.076M-tris and 0.005M-citric acid. While still fluid, the gel was covered with polythene film to prevent subsequent evaporation. The electrode vessels were filled with a borate buffer (0.3M-boric acid-0.05M-NaOH). The samples were introduced in filter paper as described by Smithies (1955). Small pieces of Whatman no. 3MM filter paper were cut to exactly the cross-section of the gel. After saturating them with the tissue extract they were inserted into slits in the gels, one-third of the way from the cathode.

*Staining for proteins.* The gels were split longitudinally and stained with Naphthalene Black 10 B (G. T. Gurr Ltd., 136 New Kings Road, London, S.W. 6) as described by Smithies (1955).

*Staining for esterases.* Gomori's (1952) modification of Nachlas & Seligman's (1949) histochemical method for esterases was adapted as follows. A stock solution containing 1% (w/v) of  $\alpha$ -naphthyl acetate in 50% (v/v) acetone was prepared. Immediately before use 0.8 ml. of this solution was added slowly with stirring to 50 ml. of water. Tetrazotized *o*-dianisidine (Fast Blue Salt B, G. T. Gurr Ltd.) (20-30 mg.) was then added to make the substrate mixture. The gels were first placed in 100 ml. of 0.25M-phosphate buffer, pH 6.25. After 15 min., freshly made substrate mixture was added and the gels were incubated at 37° until adequate development was obtained. Background staining was removed by washing the gels with methanol-acetic acid-water (50:10:50, by vol.).

In those experiments designed to investigate the inhibitory effects of physostigmine salicylate and *NNN'*-tetraisopropylpyrophosphoramidate the inhibitors were incorporated in the buffer used for making the gel. Subsequently, before staining the gels by the procedures outlined above, they were submerged for 30 min. in 0.25M-phosphate buffer, pH 6.25, containing the inhibitor. The substrate mixture was added to this as described above.

*Photography.* The gel patterns were recorded by photography on Kodak microfilm film, with standard exposure and development times determined experimentally. For photography of protein patterns a red filter was used and for photography of esterase patterns a blue filter.

*Reproducibility of the method.* An extract was prepared from the liver of a stock albino mouse and subjected to electrophoresis on four gels. The experiment was repeated on another occasion with the same extract. Identical electrophoresis patterns were obtained in all cases. Minor differences in the resolution of some bands were found with different batches of starch.

## RESULTS

In the following account the term 'zymogram' (Hunter & Markert, 1957) is used to describe the pattern obtained when a tissue extract is separated by electrophoresis on a starch gel and this is subsequently stained to reveal the location of specific enzymes.

*Relationship between distribution of esterases and protein.* When two halves of the same gel were compared, one stained for protein and the other for esterase activity, no relationship between the bands of esterase activity and the protein bands could be recognized. For instance, the fast-moving esterase band observed in most tissues preceded and did not coincide with the albumin band. Similarly, the dense, slow-moving esterase band in human liver (Fig. 2) corresponded to an area with very little protein. On the other hand, areas of dense protein staining, e.g. serum albumin, frequently corresponded with areas devoid of esterase activity.

*Esterases from different species.* Zymograms prepared from the same organs of the mouse, guinea pig, rat, fowl and human were compared. Each species had its own distinct pattern (Fig. 2). Not only was there no evidence for the occurrence of the same protein in more than one species but the number of proteins with esterase activity varied

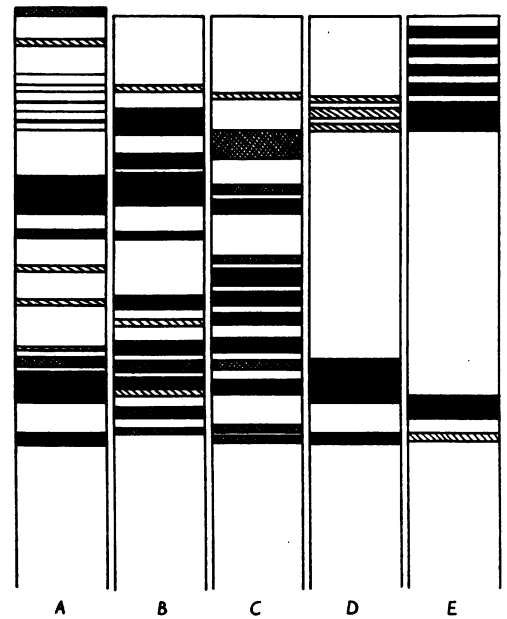


Fig. 2. Zymograms of liver extracts from different animals. In order to compare relative mobilities preparations were run in pairs in the same gels. Relative intensities of bands are indicated by shading. A, Mouse; B, rat; C, guinea pig; D, hen; E, human.

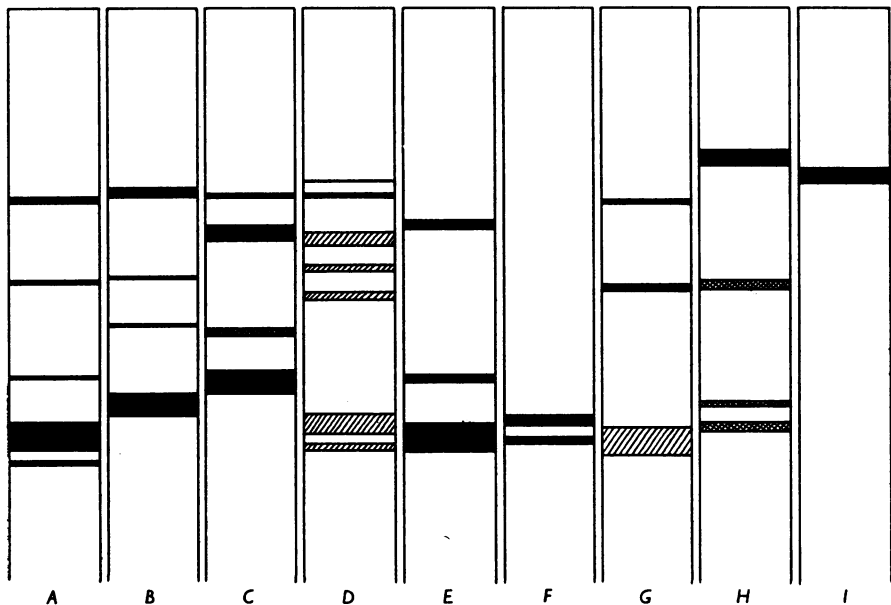


Fig. 3. Esterase zymograms from the serum of different animals. Relative intensities of bands are indicated by shading. A, Mouse; B, rat; C, guinea pig; D, human; E, rabbit; F, cow; G, lamb; H, chicken; I, horse.

very greatly from species to species. In the serum, too, as shown in Fig. 3 there were radical differences among the species examined. In all these experiments duplicate sections of the starch gels were stained for protein with Naphthalene Black 10B. Species differences were again apparent but were much less than in corresponding zymograms.

*Tissue esterases in different individuals.* Variations among individuals of the same species were investigated by preparing extracts from livers and kidneys of mice from a stock inbred strain maintained in the laboratory. In over 40 specimens the

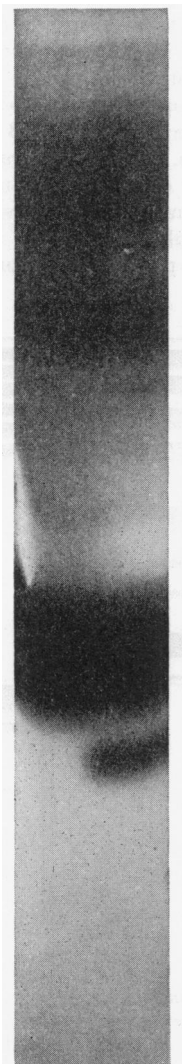


Fig. 4. Comparison of liver extracts from a normal mouse and one in which an esterase was absent. The two preparations were inserted side by side in the gel.

same sixteen bands were identified in liver preparations and the same twelve bands in kidney preparations, with only two exceptions. In one of these a single esterase was found to be missing from the liver zymogram, and in the other a single band was missing from liver and two bands (including the one missing from liver) were missing from kidney preparations. Fig. 4 demonstrates this latter case strikingly. The abnormal preparation was inserted in the same gel as a normal preparation and they were subjected to electrophoresis simultaneously.

Specimens from three adult humans and two human foetuses were also compared and identical patterns were always obtained from extracts of the same organs.

*Esterases from different tissues.* Fig. 5 represents typical zymograms obtained from extracts of liver, kidney, heart, muscle and serum of stock albino mice and illustrates that in agreement with Hunter & Markert (1957) quite different patterns are obtained from different organs in this animal. Liver exhibited a particularly complex pattern whereas at the other extreme skeletal muscle produced a simple one. Certain esterases were always found in all the mouse tissues investigated, providing a species characteristic.

Tissue from the guinea pig and rat show a similar variation from organ to organ.

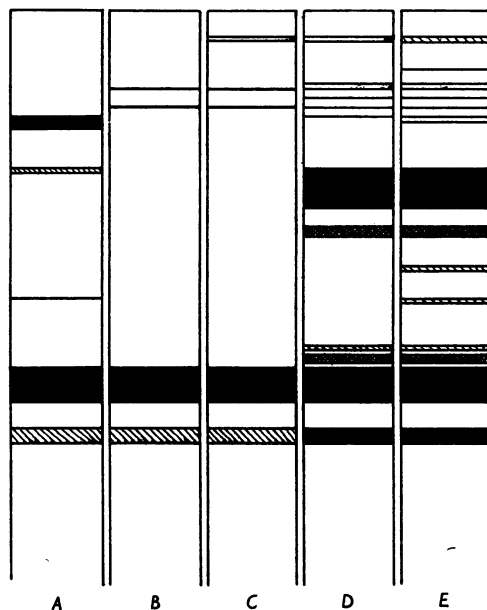


Fig. 5. Zymograms from extracts of different organs of the mouse. Relative intensities of bands are indicated by shading. A, Serum; B, skeletal muscle; C, heart; D kidney; E, liver.

In contrast, tissues from the human almost all exhibited the same pattern of seven bands. In one or two organs, notably liver, adrenal and thyroid, additional ones were observed but the differences were very minor compared with those observed in some other species (Fig. 6).

Since some variation was obtained in zymograms from different normal organs in the same animals, some tumour tissues were compared with corresponding normal tissues. In two of three human tumours, a hypernephroma and a carcinoma of the rectum, the zymograms were indistinguishable from those obtained from adrenal cortex and rectal mucosa respectively. In a third case, however, also a hypernephroma, a dense band was obtained in the zymogram which was not found in either kidney or adrenal tissue. The zymogram, in fact, most closely resembled that normally obtained from liver tissue.

*Esterases in cultured cells.* It has been suggested (Vandelli & Scaltriti, 1943) that esterases may be adaptive enzymes. However, the human pattern was retained in human cells which had been cultured *in vitro* for periods varying from a few days with kidney cells to many years with HeLa cells (Fig. 7). Further, some of the cells had been grown for at least 3 months in heterologous media containing calf serum, and cells originating from

different species still gave different zymograms although they had been cultivated in the same media for many months. These findings suggest that the patterns obtained are not dependent on the immediate environmental conditions but more probably represent differences of a genetic nature.

*Classification of esterases in zymograms.* According to Augustinsson (1958) esterases can be distinguished on the basis of substrate specificities and inhibition by specific substances. Physostigmine specifically inhibits acetylcholinesterases (C esterases) at a concentration of  $10^{-5}$  M, but this had no apparent effect on any of the esterases in zymograms from mouse liver although at  $10^{-3}$  M three bands were inhibited. In human liver, treatment with  $10^{-4}$  M-physostigmine salicylate produced a decrease in the intensity of all the bands whereas at  $10^{-3}$  M all the bands were completely inhibited. Tetraisopropyl pyrophosphoramidate specifically inhibits the ali-esterases (B esterases) as well as the C esterases. At a concentration of  $10^{-4}$  M no inhibition of any of the bands on the zymograms from human or mouse material was obtained. At  $10^{-3}$  M, partial inhibition of all the esterases of human liver and partial inhibition of one band in

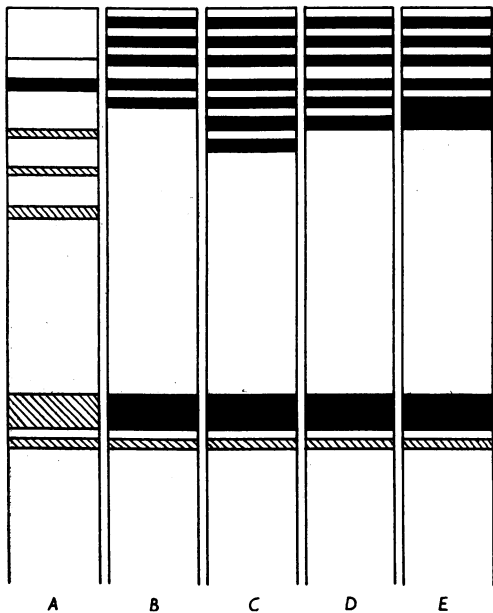


Fig. 6. Zymogram patterns obtained from different human organs. Relative intensities of bands are indicated by shading. A, Serum; B, stomach, small intestine, lung, thymus, adrenal, testis, spleen, heart; C, thyroid; D, kidney; E, liver.

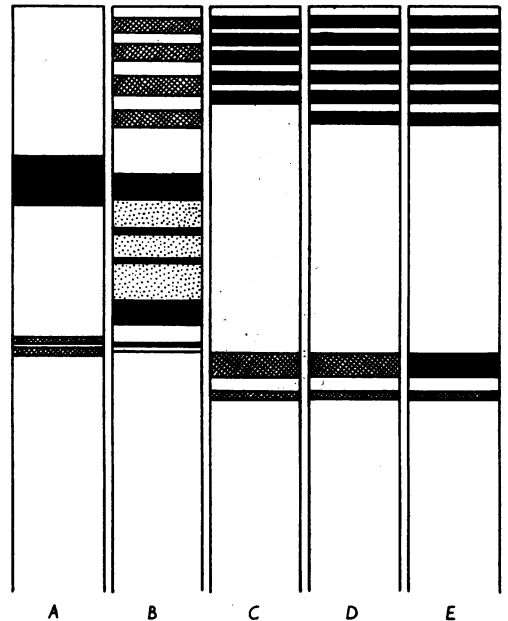


Fig. 7. Zymogram patterns from extracts of cultured cell strains compared with an extract of human kidney. Relative intensities of bands are indicated by shading. A, L 5178 Y (murine origin); B, strain L (murine origin); C, HeLa, HEP I, HLM, skin, heart (all of human origin); D, freshly cultured human-kidney cells; E, human kidney (not cultured).

mouse liver were obtained. It would seem therefore that the majority of the esterases revealed by this technique are non-specific aromatic esterases (A esterases).

### DISCUSSION

In view of the high degree of species specificity shown by the patterns from different animals and their persistence during prolonged cultivation *in vitro* the use of these techniques for the identification of cell-strains is a practical application of immediate interest.

Of more fundamental interest is the observation that a very large number of bands with esterase activity can be demonstrated by starch-gel electrophoresis. Although these bands may be artifacts caused by the adsorption of esterases on to other proteins, this explanation seems unlikely, since there is no obvious correlation between zones of high esterase activity and zones of high protein concentration. The alternative explanation is that a very large number of proteins may display esterase activity even within the same organ, at least 16 in the mouse liver. Many examples of multiple forms of enzymes have been described in recent years. These include aldolase (Taylor, Green & Cori, 1948), lactic dehydrogenase (Meister, 1950; Nielands, 1952; Markert & Møller, 1959), lysozyme (Tallan & Stein, 1953),  $\beta$ -glucuronidase (Mills, Paul & Smith, 1953), ribonuclease (Hirs, Moore & Stein, 1953), triose phosphate dehydrogenase (Krebs, 1953), enolase (Malstrom, 1957), alkaline phosphatase (Schlamowitz, 1958), peroxidase (Markert & Møller, 1959), esterases (Hunter & Markert, 1957) and trypsin (Timasheff, Sturtevant & Bier, 1956). These multiple forms of enzymes have been described within the same species and when species differences are also considered it may reasonably be assumed that an extremely large number of proteins with the same kind of activity can exist.

Proteins possessing the same enzyme activity may be related in at least three ways. First, they may represent different degrees of polymerization of the same molecular species, as is known to occur in haemocyanin (Pedersen, 1949) and insulin (Rees & Singer, 1955). The fact that esterases in different tissues of the mouse occur in almost all possible combinations in electrophoresis diagrams argues against the existence of different polymers but, on the other hand, this would provide a reasonable explanation for the differences obtained when the tissues were extracted with buffer solutions as compared with water or saline.

The second possibility is that different molecular species may exist with the same enzyme specificity. Markert & Møller (1959) have proposed the term 'isozymes' for proteins related to each other in

this way. The existence of isozymes is supported by evidence, such as that of Mills *et al.* (1953), that different proteins can be isolated with the same enzyme activity and also the findings that immunologically distinct lactic dehydrogenases (Nieselbaum & Bodansky, 1959) and phosphorylases (Henion & Sutherland, 1957) can be identified in different tissues of the same animal.

Finally, enzymes may possess different, but overlapping, specificities. Many esterases are, of course, known to fit into this category and it is well known that aromatic esters are hydrolysed by many enzymes whose specific function is quite different, e.g. chymotrypsin (Kaufman, Schwert & Neurath, 1948).

Since there is evidence that protein structure is under direct genetic control (Allison, 1959) the implication is that a considerable amount of genetic variation is compatible with performance of the same specific function. In order to reconcile this with the similarity of metabolic pathways at all levels of evolution, it is necessary to postulate a mechanism which permits very wide structural variations in a protein while at the same time maintaining function rigidly through an enormous number of generations. The most reasonable suggestion is that the structure of certain areas in an enzyme molecule are specific for enzyme function, whereas the rest of the molecule can vary widely without disturbing function significantly. There is some experimental support for this view. On the one hand, it has been shown that in different proteins with the same enzyme activity, certain areas of the molecule may be structurally similar and specific for enzyme function (Oosterbann, Jansz & Cohen, 1956; Koshland & Erwin, 1957; Oosterbann, Kunst, Van Rotterdam & Cohen, 1958; Porter, Rydon & Schofield, 1958), whereas, on the other hand, some enzyme proteins can be partially degraded without losing their functional activity (Pope, 1939; Perlmann, 1954; Richards, 1955; Harris & Li, 1955; Rogers & Kalnitsky, 1957; Hill & Smith, 1958).

It follows that there may be an infinite variety of protein molecules with the same functional activity. It is not surprising therefore that a great multiplicity of species-specific and even tissue-specific enzymes are revealed by starch-gel electrophoresis, an analytical tool with a resolving power which approaches that of immunological methods.

### SUMMARY

1. The separation of tissue esterases by starch-gel electrophoresis is described.
2. Different species exhibit entirely different patterns by this technique and these are highly reproducible.

3. In some species considerable differences can be recognized between different tissues, although underlying species characteristics can always be recognized.

4. The patterns persist in cells which have been cultured *in vitro* for many years and are therefore genetically stable.

5. It is concluded that an enormous number of proteins with the same functional activity can exist and the significance of this observation is discussed.

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*Biochem. J.* (1961) **78**, 424

## Anti-Inflammatory Drugs and Intermediary Metabolism

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Salicylate uncouples oxidative-phosphorylation reactions in respiring mitochondrial preparations (Brody, 1956), and many of its effects on the meta-

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bolism of animals and isolated tissues are explicable in terms of this action (Smith, 1959; Smith & Moses, 1960). However, the anti-inflammatory properties of salicylate must be mediated by mechanisms other than an uncoupling action, since 2:4-dinitrophenol, although a more powerful uncoupling reagent than salicylate, lacks anti-