CHROM. 12,896

FRACTIONATION AND IDENTIFICATION OF HEPARIN AND OTHER ACIDIC MUCOPOLYSACCHARIDES BY A NEW DISCONTINUOUS ELECTROPHORETIC METHOD

PIETRO BIANCHINI, HELENA B. NADER, HELIO K. TAKAHASHI, BRUNA OSIMA, ANITA H. STRAUS and CARL P. DIETRICH[*,*]

Opocrin Laboratories, Modena (Italy) and Depto. de Bioquimica, Escola Paulista de Medicina, Caixa Postal 20372, 01000-São Paulo-SP (Brazil)

(First received March 11th, 1980; revised manuscript received April 22nd, 1980)

SUMMARY

A new discontinuous electrophoretic method for fractionation of heparin into two or three different components and of these from other acidic mucopolysaccharides is described. The method consists of electrophoresis first in agarose in barium acetate and then in diaminopropane buffer in the same direction. A combination of this discontinuous system with barbital buffer in a two-dimensional electrophoresis for identification of mucopolysaccharides is also described.

INTRODUCTION

Heparin is a mixture of molecules differing in their molecular weight[1,2] and chemical complexity[3,4], and having monomeric and dimeric forms[5]. The characterization of this family in tissues involves methods such as electrofocusing[1] and affinity chromatography[4], as well as chemical and enzymatic analysis[6,7].

Several simple methods have been developed to differentiate the heparin family from the other acidic mucopolysaccharides (AMPS). The most widely used involves electrophoresis coupled with divalent cations such as those of barium[8], zinc[9], calcium[10] and copper[11], and with diamines[12]. It was apparent from these studies that only a combination of two electrophoretic systems could resolve the heparins from the other AMPS[12].

Recently, a new method using electrophoresis in barium acetate has been employed to differentiate heparin and other AMPS[13]. In trying to reproduce this method, we have observed that most heparins, when extracted from either tissues or commercial preparations, are fractionated into at least three different components, one of them co-migrating with heparitin sulphate.

---

* Address: Depto. de Bioquímica, Escola Paulista de Medicina, Caixa Postal 20372, 01000-São Paulo-SP, Brazil.

The present paper describes the fractionation of heparin and its resolution from the other AMPS by a new discontinuous electrophoretic method.

MATERIALS AND METHODS

Mucopolysaccharides and other reagents

Chondroitin sulphates A, B and C were purchased from Miles Labs. (Elkhart, IN, U.S.A.). Heparitin sulphate was prepared as previously described[14]. Heparin from different commercial sources was kindly provided by UpJohn Co., Lederle, Abbot, Riker, Fisher, Opocrin and Laob laboratories. Sulphated mucopolysaccharides were kindly donated by Professor M. B. Mathews, University of Chicago. 1,3-Diamino-propane was purchased from Aldrich (Milwaukee, WI, U.S.A.). 5,5-Diethylbarbi-turic acid (barbital) and barium acetate were obtained from E. Merck (Darmstadt, G.F.R.), and agarose (standard-low$_{mr}$) from Bio-Rad Labs. (Richmond, CA, U.S.A.).

Agarose gel electrophoresis of AMPS

Agarose gel slides (7.5 × 5.0 cm, 0.1 cm thick) were prepared with 0.5% agarose in 0.06 M barbital buffer (pH 8.6), in 0.04 M barium acetate (pH 5.8) or in 0.1 M 1,3-diaminopropane acetate buffer (pH 9.0). Samples (3–5 μl) containing 1–10 μg of AMPS were applied to the agarose gel slide in slots of 0.3 cm at a distance of 1 cm from the edge of the slide. The slots were made with small pieces of Whatman No. 3MM filter-paper (1 × 0.3 cm), which were inserted into the gel such that they were perpendicular to its surface. The agarose slide was then subjected to electro-phoresis at 5°C in a chamber similar to that described by Wieme[15].

For the discontinuous electrophoresis the following procedure was used. The samples were applied to agarose slabs prepared in 0.04 M barium acetate and sub-jected to electrophoresis for 10 min at 75 V (45 mA) in a chamber prepared with the same buffer at 5°C. The whole gel slab (which is mounted on a glass microscope slide[16]) was then transferred to another chamber which had been prepared with the diaminopropane acetate buffer and maintained at 5°C for 15 min. The current (100 V) was then applied in the same direction (towards the positive electrode) for 90 min or until the dye indicator (cresol red) had migrated 4.5 cm from the origin. The barium ions migrate from the gel in a sharp front and are replaced by diamino-propane ions. This replacement (which is complete in 30–40 min) is easily observed by the change of colour of the cresol red, from yellow (pH 5.8 of the barium acetate buffer) to red (pH 9.0 of the diaminopropane acetate buffer). After electrophoresis, the gel was immersed in a 0.1% solution of Cetavlon (cetyltrimethylammonium bro-mide; E. Merck) for 3 h. The gel was then covered with a Whatman No. 3MM filter-paper strip that had previously been wetted with the Cetavlon solution, and the slides were placed under a 250-W infrared lamp for 2 h in a current of air from a fan. They were then stained with a 0.1% solution of toluidine blue in acetic acid–ethanol–water (0.1:5:5). The slides were de-stained with the same solution but without toluidine. Further details on the preparation of the chambers and slides are given elsewhere[16].

The two-dimensional agarose gel electrophoresis was performed as follows. The mixtures of AMPS were applied in the agarose gel and subjected to electro-phoresis in the discontinuous barium–diaminopropane system as described above. After the run, agarose gel strips (5 × 0.2 cm) containing the fractionated AMPS

were cut off and placed in a slot of the same width and length situated transversely 2 cm from the edge of another agarose gel slide prepared with the other buffer systems. The slide was then subjected to electrophoresis for 30 min at 100 V and stained as described above. In some instances a two-dimensional agarose polyacryl-amide electrophoresis was performed. After the run in the agarose system the gel strip containing the fractionated AMPS was cut off and placed in a slot of the same width and length situated transversely 2 cm from the edge of the polyacrylamide gel slab prepared in barbital buffer. The slide was then subjected to electrophoresis for 30 min at 100 V, stained with a 0.1 % toluidine blue solution in 1 % acetic acid and destained with the same solution but without the dye. Cellulose acetate electro-phoresis was performed essentially as described by Cappelletti et al.[13]. Polyacryl-amide gel electrophoresis was performed as previously described[14].
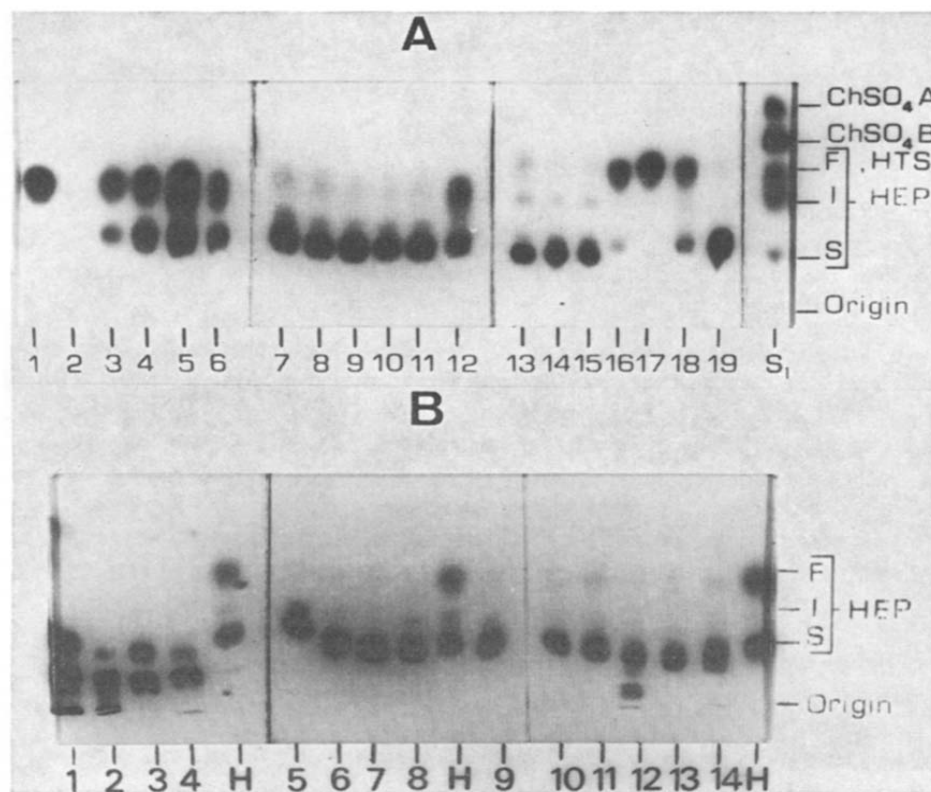

Fig. 1. Agarose gel electrophoresis of different heparin preparations in the discontinuous buffer. A, Commercial heparin preparations (10 μg): 1 = Fisher; 2 and 3 = Lederle; 4 = Riker; 5 = BDH; 6 = Laob; 7–11 and 13–15 = UpJohn batches; 12 = Laob; 16 = Lederle; 17 = Fisher; 18 = Laob; 19 = standard from Professor Mathews; S_1 = mixture of mucopolysaccharides. B, Heparin extracted from different tissues (10 μg) as described in ref. 7: 1 = rat skin; 2 = rat lung; 3 = rat thymus; 4 = mouse skin; 5 = bovine fetus liver; 6 = bovine fetus lung; 8 = bovine fetus ileum; 9 = bovine ileum; 10 = bovine thymus; 11 = pig lymph node; 12 = cat lung; 13 = dog spleen; 14 = human ileum; H = commercial heparin (Laob). For abbreviations, see Fig. 4.

RESULTS AND DISCUSSION

Fractionation of heparin in the discontinuous barium–diaminopropane agarose gel electrophoresis

The agarose gel electrophoresis in barium–diaminopropane buffers of nineteen commercial heparin preparations is shown in Fig. 1A. Most of the heparins are fractionated into at least three components. However, the relative proportions of the fractions vary among heparin preparations. For instance, reference heparin obtained from Professor Mathews contains almost exclusively the slow-moving component, whereas heparin from Fisher Co. contains only the fast-moving one. Other heparins from Opocrin, Laob, UpJohn, Abbot and Lederle contain the slow- and fast-moving components plus another fraction with intermediate migration rate. Thirty-five other commercial preparations contain at least the two main components in varying proportions, including the heparin international standard (WHO-III) which contains 63% of the slow- and 37% of the fast-moving component. Several heparins obtained from different tissues also show the presence of the two main fractions in the discontinuous buffer (Fig. 1B). The relative proportions of the two main fractions are constant regardless of the heparin concentration as shown in Fig. 2. This figure also shows that the optical density is proportional to the heparin concentration up to 10 μg. In order to substantiate the presence of the two fractions a two-dimensional agarose gel electrophoresis in the same discontinuous system was performed as shown in Fig. 3A. It is clear that there was no further fractionation of the two heparin components.
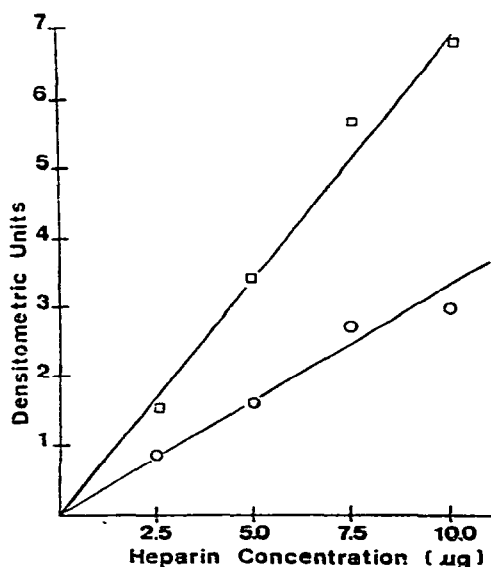

Fig. 2. Optical density of international standard heparin in discontinuous agarose gel electrophoresis. Different concentrations of WHO-III international heparin standard were subjected to electrophoresis under the conditions described in Materials and methods. O, Fast heparin component; □, slow heparin component.
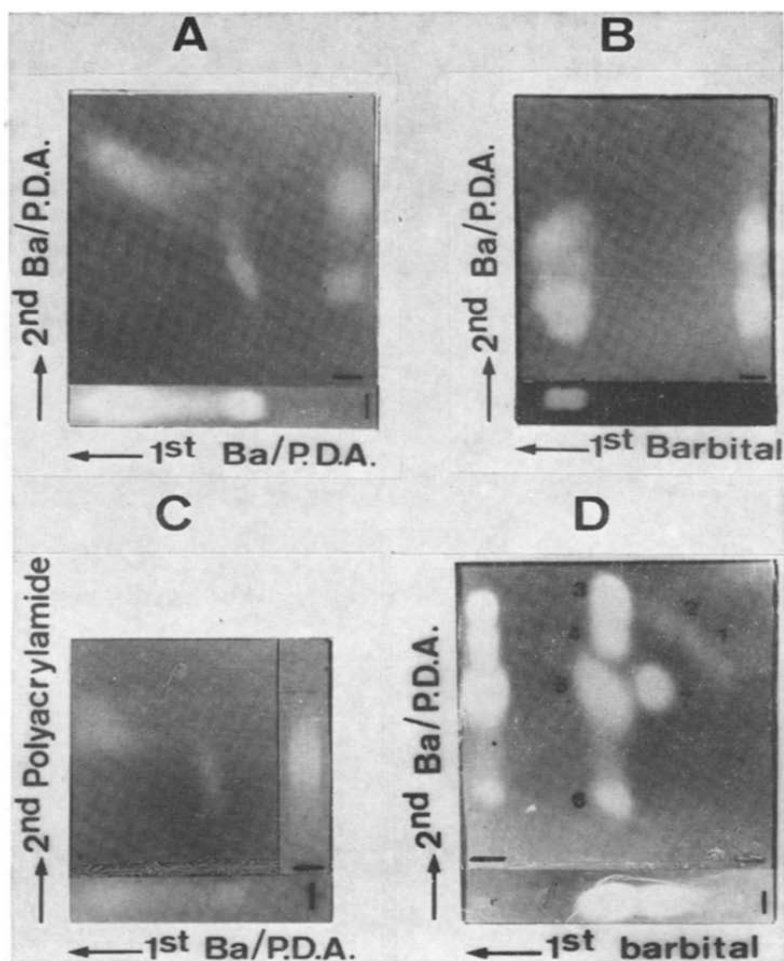
Fig. 3. Two-dimensional gel electrophoresis of heparin and other sulphated mucopolysaccharides. A, Two-dimensional electrophoresis of heparin in barium–diaminopropane discontinuous buffer; first and second dimension with the same buffer. B, Two-dimensional electrophoresis of heparin in barbital (first dimension) and discontinuous barium–diaminopropane (second dimension) buffers. C, Two-dimensional electrophoresis of heparin in discontinuous barium–diaminopropane (first dimension) and polyacrylamide gel (second dimension). D, Two-dimensional electrophoresis of a mixture of heparin and acidic mucopolysaccharides in barbital (first dimension) and discontinuous barium–diaminopropane buffers (second dimension): 1 = hyaluronic acid; 2 = kerato sulphate; 3 = chondroitin sulphate A; 4 = chondroitin sulphate B; 5 = heparin (fast-moving component); 6 = heparin (slow-moving component); 7 = heparitin sulphate. P.D.A. = diaminopropane acetate buffer.

Parameters involved in the fractionation of heparin by the discontinuous method.

    Some heparins also yield two bands when subjected to electrophoresis in barbital buffer. To determine whether the fractionation of heparin in the discontinuous system is related to its fractionation in barbital, a two-dimensional electrophoresis was performed using these two buffer systems. Fig. 3B shows that each of the two bands of the barbital system gives rise to two new bands in the discontinuous
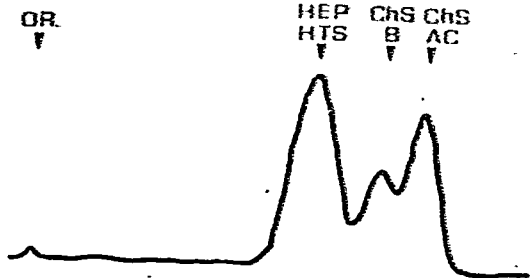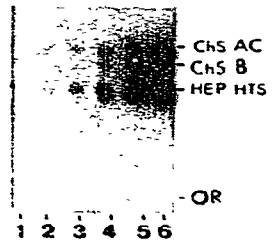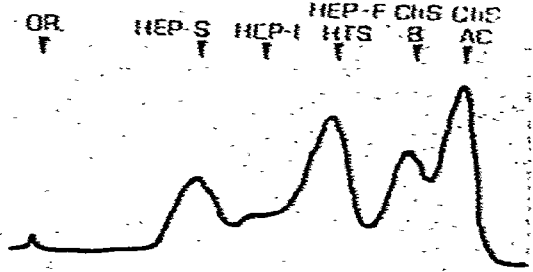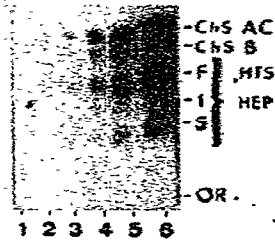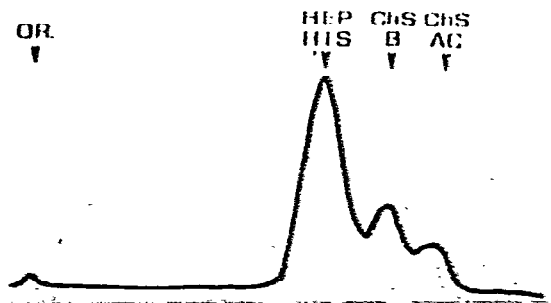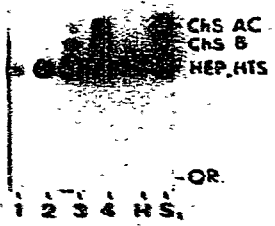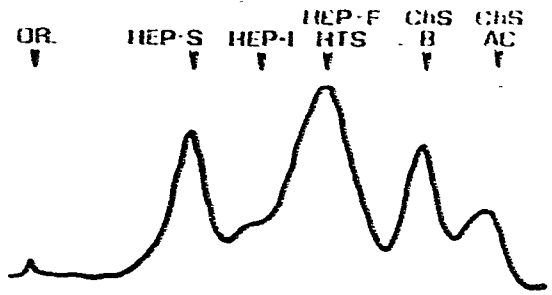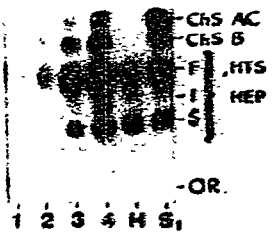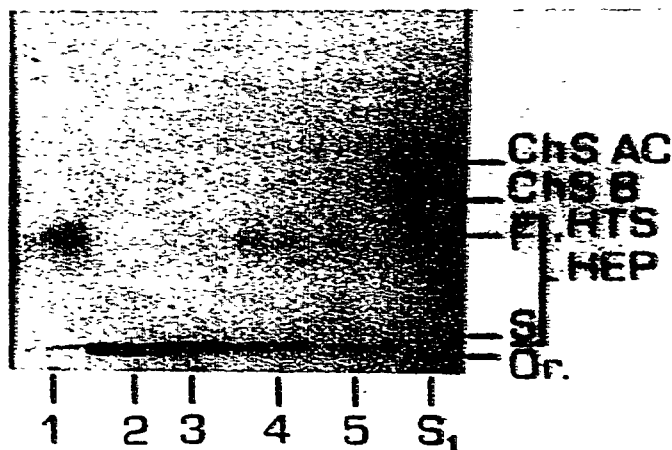
Fig. 5. Cellulose acetate electrophoresis of some heparin preparations. The experiment was performed as described in ref. 13. Heparin preparations: 1 = Fisher; 2 = Professor Mathews; 3 = UpJohn; 4 = Lederle; 5 = Laob; S₁ = standard mixture of chondroitin sulphates A and C (ChS AC), B (ChS B) and heparitin sulphate (HTS).

buffer, indicating that the fractionation of heparin in the two systems are in-dependent phenomena.

A two-dimensional electrophoresis in discontinuous buffer in agarose and polyacrylamide gels is shown in Fig. 3C. It seems that the fractionation of heparin in the discontinuous system is proportional to molecular weight since the two fractions exhibit a clear difference of migration in polyacrylamide gel electrophoresis.

Fractionation of heparin and other acidic mucopolysaccharides

Fig. 4 shows the electrophoresis in the discontinuous buffer and in diamino-propane buffer of a mixture of heparin, chondroitin sulphates A and B and heparitin sulphate in different concentrations, as well as of a mixture of mucopolysaccharides extracted from bovine stomach. A better separation of the AMPS is obtained in the new system when compared with the conventional diaminopropane buffer. These two buffers nevertheless are unable to resolve the fast-moving heparin component from heparitin sulphate. This experiment also shows that the limit of detection of the AMPS is around 0.3–0.5 μg and these compounds can easily be estimated by densitometry. Fig. 5 shows the cellulose acetate electrophoresis in barium acetate under the conditions described by Cappelletti *et al.*[13]. Again a co-migration of heparitin sulphate and the fast-moving heparin component is observed. The heparin

Fig. 4. Agarose gel electrophoresis of heparin and sulphated mucopolysaccharides. A, A mixture of heparin, chondroitin sulphates A and B and heparitin sulphates at concentrations of 0.1 (1), 0.3 (2), 0.5 (3), 1 (4), 5 (5) and 10 (6) μg in 5 μl of water as subjected to electrophoresis in the discontinuous buffer (upper) and in the diaminopropane acetate buffer (lower). B, Sulphated mucopolysaccharides extracted from bovine stomach at concentrations of 5 (1), 10 (2), 20 (3) and 50 (4) μg were subjected to electrophoresis in the discontinuous buffer (upper) and in the diaminopropane acetate buffer (lower). OR = Origin; ChS AC = chondroitin sulphates A and C; ChS B = chondroitin sulphate B; HTS = heparitin sulphate; HEP = heparin; F = fast-moving component; I = intermediate component; S = slow-moving component. H = 10 μg of heparin; S₁ = a mixture of 20 μg each of sulphated mucopolysaccharides.

standard used by the authors was the one prepared by Professor Mathews and which does not contain the fast-moving component (Fig. 1A). This standard seems to be an exception among more than 50 heparin preparations tested and care should be exercised in the use of this methodology alone for the identification of heparins.

The complete resolution of all the AMPS could be obtained only by a two-dimensional electrophoresis in barbital buffer (first dimension) and discontinuous buffer (second dimension) as shown in Fig. 3D.

Molecular weight seems to play an important rôle in the fractionation of heparin in this discontinuous electrophoretic buffer system. The results also suggest that the other AMPS complex to different degrees with the barium, which explains the better separation when compared to the regular diaminopropane buffer.

ACKNOWLEDGEMENTS

REFERENCES

1 H. B. Nader, N. M. McDuffie and C. P. Dietrich, *Biochem. Biophys. Res. Commun.*, 57 (1974) 488.
2 N. M. McDuffie, C. P. Dietrich and H. B. Nader, *Biopolymers*, 14 (1975) 1473.
3 R. D. Rosenberg, G. Armand and L. Lam, *Proc. Nat. Acad. Sci. U.S.*, 75 (1978) 3065–3069.
4 U. Lindahl, G. Backstrom, M. Hook, L. Thunberg, L. Fransson and A. Linker, *Proc. Nat. Acad. Sci. U.S.*, 76 (1979) 3198.
5 C. P. Dietrich, H. B. Nader and N. M. McDuffie, *An. Acad. Brasil. Ciênc.*, 47 (1975) 301.
6 M. E. Silva and C. P. Dietrich, *J. Biol. Chem.*, 250·(1975) 6841.
7 H. B. Nader, H. K. Takahashi, A. H. Straus and C. P. Dietrich, *Biochim. Biophys. Acta*, 627 (1980) 40.
8 E. Wessler, *Anal. Biochem.*, 26 (1968) 439.
9 R. S. Prout, *Biochim. Biophys. Acta*, 177 (1969) 157.
10 N. Seno, K. Anno, K. Kondo, S. Nagase and S. Saito, *Anal. Biochem.*, 37 (1970) 197.
11 R. Hata and Y. Nagai, *Anal. Biochem.*, 45 (1972) 462.
12 C. P. Dietrich, N. M. McDuffie and L. O. Sampaio, *J. Chromatogr.*, 130 (1977) 299.
13 R. Cappelletti, M. Del Rosso and V. P. Chiarugi, *Anal. Biochem.*, 99 (1979) 311.
14 C. P. Dietrich and H. B. Nader, *Biochim. Biophys. Acta*, 343 (1974) 34.
15 R. J. Wieme, *Agar Gel Electrophoresis*, Elsevier ,Amsterdam, 1965.
16 L. B. Jaques, R. E. Ballieux, C. P. Dietrich and L. W. Kavanagh, *Can. J. Physiol. Pharmacol.*, 46 (1968) 351.