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O-METHYLATION OF ^{3}H-NOREPINEPHRINE BY EPIDIDYMAL ADIPOSE TISSUE*

GEORGE J. TRAIGER† and DEANE N. CALVERT

Department of Pharmacology, Marquette School of Medicine, Milwaukee, Wis., U.S.A.

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Abstract—The presence of catechol-*O*-methyl transferase (COMT) activity in rat epididymal adipose tissue preparations was studied *in vitro* with ^{3}H-norepinephrine as substrate. A radioactive metabolite which behaved chromatographically like normetanephrine (NM) was isolated from the incubation medium. It was possible to inhibit the formation of this metabolite by the addition of pyrogallol or of nonradioactive NM to the incubation medium. Whole epididymal adipose tissue had greater COMT activity than did isolated adipocytes containing an equivalent amount of triglyceride. Centrifugation of adipocyte homogenates revealed that twice as much COMT activity was in the 30,000 *g* particulate as in the supernatant fractions. This enzyme activity was further found to reside in preparations of adipocyte plasma membranes. These observations suggest that adipocyte COMT exists partially in a particle-bound form, probably associated with the cell membrane.

IN THE PROCESS of changing our experimental design from the use of whole pieces of adipose tissue to the use of isolated adipose cells in the study of hormone-stimulated lipolysis, we noted that norepinephrine (NE) appeared to be more potent in the cell preparation than in the whole tissue preparation. A similar observation had previously been made by Rodbell[1] in his study of ACTH-stimulated lipolysis. Among the possible explanations for this potency difference in the case of NE is a difference in the metabolic disposition of the amine in the two preparations. Since isolated adipose cells are devoid of sympathetic nerves, blood vessels and connective tissue which are present in whole tissue, one might expect such a difference. To evaluate this possibility, a study of the enzyme, catechol-*O*-methyl transferase (COMT, EC 2.1.1.6), in various adipose tissue preparations was undertaken. Axelrod and Tomchick[2] have shown that COMT is present in the soluble portion of a homogenate of most tissues, but its presence in the 30,000 *g* supernatant of a homogenate of epididymal adipose tissue could not be detected by Stock and Westermann[3] using a fluorometric procedure for the determination of normetanephrine. By using the more sensitive procedure of the measurement of ^{3}H-normetanephrine formation from ^{3}H-NE, we have found that the enzyme is present in epididymal adipose tissue and cells isolated from such tissue. Moreover, much of this activity appears to be particle bound in these isolated cells. A preliminary report of these findings has been published.[4]

MATERIALS AND METHODS

Male Holtzman rats weighing 240–350 g were sacrificed by decapitation and care-

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† Predoctoral Trainee, United States Public Health Service TI GM-370.

fully exsanguinated. The epididymal fat pads were rapidly excised and weighed and fat cells were prepared by a modification of the procedure of Rodbell, which has been previously described.[5]

For the preparation of cell fractions, adipocytes were homogenized in 0·1 vol. of cold isotonic KCl and centrifuged at 30,000 *g* for 30 min. The infranatant fractions were removed by aspiration and kept at 0° until further use. Particulate fractions were resuspended in a suitable volume of isotonic KCl to give a protein concentration of from 1·7 to 2·4 mg/ml and added to the incubation medium. Fat cell "ghosts" were prepared by the method of Rodbell,[6] with the exception that Na-ATP was substituted for Tris-ATP in the hypotonic lysing medium. Protein was determined in the various tissue preparations by the method of Lowry *et al.*[7] Triglyceride was determined by the method of Van Handel and Zilversmit.[8]

In experiments measuring normetanephrine (NM) formation by epididymal adipose tissue or isolated fat cells, approximately 3 g of epididymal tissue or cells derived from 3 g of tissue were incubated in 5 ml Krebs bicarbonate buffer containing 0·12 μmole *l*-norepinephrine bitartrate, 0·76 μmole S-adenosyl-L-methionine iodide, 0·59 to 0·89 μc *dl*-norepinephrine-7-^{3}H acetate, 29·5 μmole $MgCl_2$, 0·27 μmole Na_2EDTA, 1·13 μmole ascorbic acid, and 0·30 μmole phosphate buffer. Final volume of the incubation mixture prior to tissue or cell addition was 6·2 ml at pH 7·2. The tritiated norepinephrine was purified before use by adsorption on an alumina column at pH 8·4, washing with 30 ml of distilled water and eluting with 10 ml of 0·2 N HCl. Incubations were for 1·5 hr at 37° in a Dubnoff metabolic shaker.

^{3}H-normetanephrine was isolated from the incubation medium by the method of Iversen *et al.*[9] Briefly, this method consists of adsorption of the catecholamines on an alumina column, passage of the effluent containing the methoxylated metabolites through a column of Dowex 50W-X4 in the sodium form, and elution of the methoxylated amines from the column with an ethanol–HCl mixture. Blank incubations, in which no tissue was added to the incubation medium, indicated that a small amount of ^{3}H-norepinephrine (0·40 per cent) distributes on the columns like the methylated compounds, and all values were accordingly corrected for this blank. Total radioactivity present in the medium after incubation was almost entirely recovered. Aliquots of all column fractions were counted in a Packard Tri-Carb liquid scintillation spectrometer after the addition of a Triton-X-100, toluene phosphor solution[10] (maximum counting error 2 per cent). Automatic external standardization was used for determining and correcting quench.

For the TLC procedures, the ^{3}H-normetanephrine in the Dowex-50 eluate was extracted into isoamyl alcohol[11] and evaporated under vacuum. The residue was taken up in a small volume of ethanol and spotted at the origin of TLC glass plates, prepared with polyamide (0·25 mm). The plates were developed in a solvent system consisting of isobutanol:glacial acetic acid: cyclohexane (8:0·7:1) for a period of 3 hr.[12] After development, authentic normetanephrine and norepinephrine were located by spraying the chromatographs with trichloro-*p*-benzoquinonemine.[12] One square cm increments of the polyamide coating were scraped into scintillation vials to which 0·1 ml acidic methanol was added and the radioactivity was determined

l-Norepinephrine bitartrate was obtained from Winthrop Laboratories, New York, N.Y.; S-adenosyl-L-methionine iodide from Calbiochem, Los Angeles, Calif.; *dl*-norepinephrine-7^{3}H acetate, sp. act. 0·28 c/m-mole, from New England Nuclear,

Boston, Mass.; Triton X-100 from Rohm & Hass, Philadelphia, Pa.; polyamide from Woehlm, New Orleans, La.; all other chemicals were of highest purity commercially available.

RESULTS

Initial experiments in which ^{3}H-norepinephrine was incubated with either adipose tissue or isolated adipose cells indicated that a substance was being formed which behaved similarly to normetanephrine (NM) during the chromatographic separation techniques.[9] To further substantiate this, the eluates from the Dowex-50 column were subjected to thin-layer chromatography by a method which allows identification of small amounts of catecholamines and of the various metabolites of the amines.[12] Fig. 1 is a plot of the cpm of the scrapings of 1-cm increments from the thin-layer plates against centimeters from the origin. It is clear that both the whole tissue and the isolated cells formed a radioactive material which had identical R_f values on these plates. This R_f (0·63) is also identical with that of authentic normetanephrine run in the same system. Because a small amount of ^{3}H-NE is present in the eluate from the Dowex column containing the normetanephrine fraction, it was deemed possible that the strongly alkaline conditions for extracting normetanephrine could be producing an artifactural material originating from this NE and that such material could have an R_f similar to that of NM. To examine this possibility, the procedure for the extraction of NM was conducted with an amount of ^{3}H-NE equal to that present in the pooled Dowex eluates of the tissue preparations. The residue was chromatographed in the same system as the NM fraction. The radioactivity detected in this procedure is equivalent to instrument background and is shown in Fig. 1.

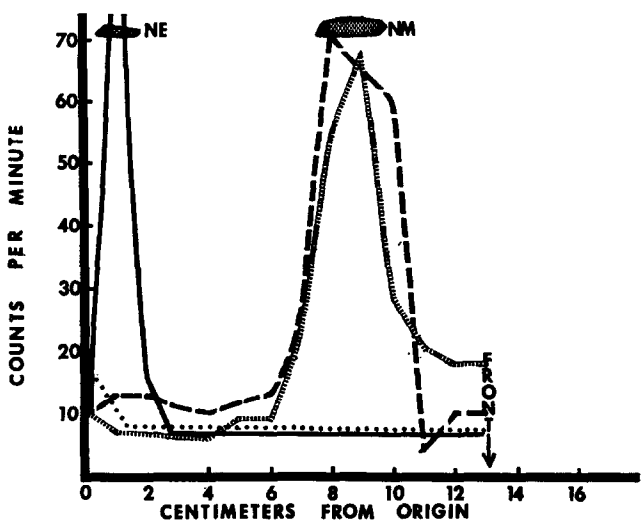

FIG. 1. Chromatographic profile of ^{3}H-NE and its metabolite isolated from the incubation medium of adipose tissue preparations. The Dowex eluates of several experiments were pooled and chromatographed simultaneously with authentic NE and NM, as described in Methods. Adipocyte metabolite (— — — —); epididymal tissue metabolite (ıılıılı); ^{3}H-NE (——); residue obtained after the procedure for the extraction of NM was conducted with ^{3}H-NE (.....). The shaded areas labelled NE and NM represent the location of authentic norepinephrine and normetanephrine chromatographed with the extracts from the incubation media.

As a further check on the identification of the tritiated metabolites and upon the use of the column and chromatographic procedures in our hands, the 30,000 *g* supernatant fraction of a rat liver homogenate, a known source of COMT, was incubated with all the components of incubation medium and subjected to the same chromatographic procedures. Approximately 37% of the total radioactivity was found to be converted to NM and, as shown in Fig. 2, displayed a radioactive peak on the thin-layer plates identical with the material formed by adipocytes, by isolated tissue preparation and with authentic NM.

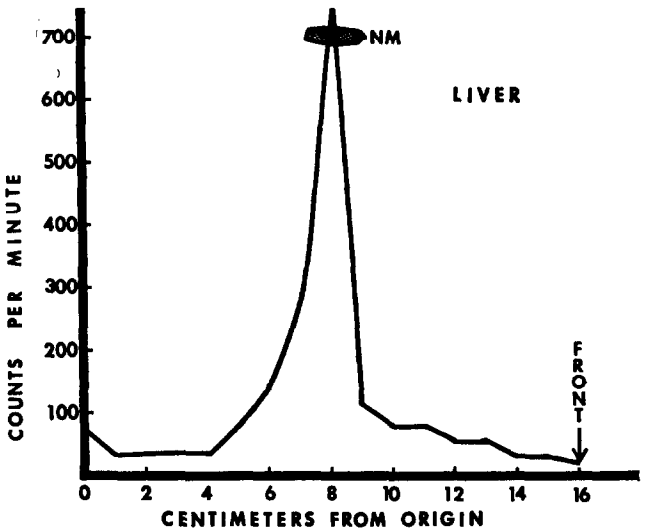

FIG. 2. Chromatographic profile of tritiated metabolite isolated after incubation of ^{3}H-NE with the 30,000 *g* supernatant fraction of a rat liver homogenate. The shaded area labeled NM represents the location of authentic normetanephrine chromatographed with the extracts from the incubation medium.

TABLE 1. INHIBITION *IN VITRO* OF COMT ACTIVITY IN ADIPOCYTE PATRICULATE FRACTIONS

Additions	N	Normetanephrine* formed (n-moles/mg protein/hr)	Per cent inhibition	P value
l-Norepinephrine $3–10 \times 10^{-6}$ M	4	0·097 ± 0·007		
l-Norepinephrine 3×10^{-6} M + Normetanephrine 1×10^{-3} M	4	0·015 ± 0·004	89	<0·001†
l-Norepinephrine $1–2 \times 10^{-5}$ M	4	0·093 ± 0·009		
l-Norepinephrine $1–2 \times 10^{-5}$ M + Pyrogallol 1×10^{-3} M	4	0·031 ± 0·008	67	<0·02‡

* Mean ± S.E.
† Group comparison.
‡ Paired comparison.

As additional evidence that the metabolite being formed by adipose tissue was NM, attempts were made to inhibit its formation by incubating an adipose tissue fraction in the presence of COMT inhibitors. The 30,000 *g* particulate from a homogenate of adipose cells was used as the source of COMT, since we had previously observed that the major share of the activity resided here.[4] High concentrations of NM have been shown by Allen and Lum[13] to inhibit COMT *in vitro*. This effect probably represents product inhibition of the enzyme reaction. As shown in Table 1, the addition of 10^{-3} cold NM to the 30,000 *g* particulate from an adipocyte homogenate effected an 89 per cent reduction in the formation of ^{3}H-NM. Pyrogallol, a competitive inhibitor of COMT, was also found to inhibit the formation of the tritiated metabolite by adipocyte particulate fractions. In these experiments, the resuspended pellet was divided into two equal fractions, one being incubated in the presence of 10^{-3} M pyrogallol and the other serving as a control. Table 1 illustrates that this concentration of pyrogallol produced 68 per cent inhibition of the production of the tritiated metabolite.

Incubation of isolated adipocytes or whole fat pads showed that approximately 2–5 per cent of the total radioactivity, depending on the tissue preparation, behaved as a methoxylated amine in the column separation procedures. Fig. 3 demonstrates the difference in the rate of NM formation by isolated adipocytes and by whole epididymal fat pads. Pairs of epididymal fat pads were separated, one being used for a whole tissue incubation and the other for the preparation of isolated cells. By using this procedure the triglyceride content of the adipocytes was found to be 86 per cent of the epididymal tissue from which they were isolated. As shown in Fig. 3, the rate of NM formation in isolated cells is only 58 per cent of that found in whole tissue, the difference being significant at the 0·01 per cent level.

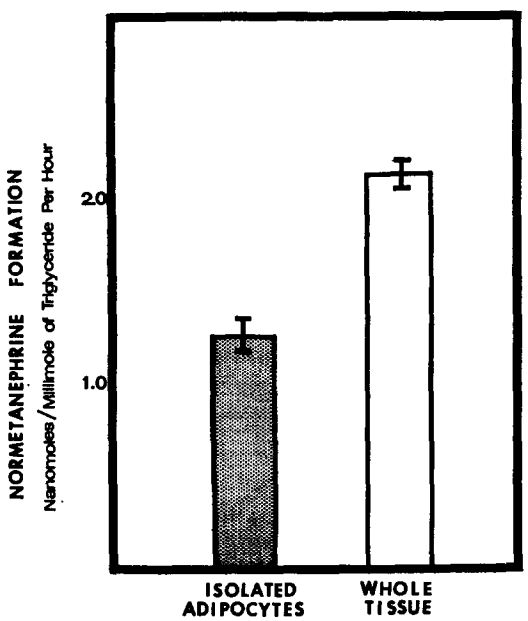

FIG. 3. Experimental conditions are described in Methods. The values shown are the means of 4 experiments. The vertical lines represent ± 1 S.E.

Table 2 demonstrates the sedimentation characteristics of the COMT activity residing in isolated fat cell homogenates. Axelrod and Tomchick[2] have shown that COMT is in the soluble fraction of liver and of other organs in which its presence can be demonstrated. However, incubation of the 30,000 *g* supernatant of an adipocyte homogenate produces only 28 per cent of the total activity found in intact cells, the specific activity also decreasing to 68 per cent of the transformation by whole cells.

TABLE 2. NORMETANEPHRINE FORMATION BY ADIPOCYTE PREPARATIONS*

Adipocyte preparation	N	Total activity: Normetanephrine formed/g adipose tissue/hr (n-moles)	Sp. act.: Normetanephrine formed/mg protein/hr (n-moles)
Intact adipocyte	4	0·300 ± 0·049	0·069 ± 0·007
Adipocyte homogenate	2	0·265 ± 0·015	0·067 ± 0·024
30,000 *g* Supernatant of adipocyte homogenate	4	0·083 ± 0·026	0·048 ± 0·014
30,000 *g* Particulate of adipocyte homogenate	4	0·159 ± 0·031	0·093 0·009

* All values represent the mean ± S.E.

Whole homogenates form NM at rates approximately equal to intact cells, indicating that activity is not destroyed during homogenization. In an attempt to locate the apparent loss of enzyme activity, the 30,000 *g* pellet was resuspended and used as the tissue preparation. As shown in Table 2, the specific activity of the cell particulate is approximately 2-fold greater than that of the supernatant, the difference being highly significant ($P < 0{\cdot}005$). Summation of the activity found with the supernatant and particulate fractions is in good agreement with that found for adipocyte homogenates. These results imply that a major fraction of adipocyte COMT activity exhibits sedimentation characteristics suggestive of a particle-bound enzyme.

Table 3 attempts to identify the cellular component responsible for COMT activity. While the major share of activity does reside in the 30,000 *g* pellet of an adipocyte homogenate, a lesser, though significant amount remains in the soluble fraction. It was possible to inhibit the supernatant activity by the addition of 10^{-3} M normetanephrine. Attempts to delineate the cellular component by differential centrifugation met with limited success. Results were greatly affected by the degree of homogenization, extensive homogenization producing a slurring of the COMT activity throughout the various centrifugal fractions. Yet, the greatest activity was usually found associated with the pellet. Thus, if the COMT activity present in adipocytes were particle bound, Rodbell's procedure for the preparation of adipocyte ghosts seemed to offer a suitable tissue preparation to investigate this finding. As shown in Table 3, the COMT activity was found to reside principally in the sacs of plasma membrane, sedimenting at 900 *g*. The 10,000 *g* pellet, containing 75 per cent of the mitochondria,[6] exhibited only a minor amount of the total activity. As shown in experiment 3, membrane activity could be markedly diminished with 10^{-3} M NM.

DISCUSSION

Although the work of Stock and Westermann[3] suggested that epididymal adipose tissue is lacking the enzyme, catechol-*O*-methyl transferase, some unpublished experiments which we had conducted showed that pyrogallol was capable of potentiating the lipolytic activity of norepinephrine on adipose cells. Utilization of the more sensitive method of detecting COMT, the formation of ^{3}H-normetanephrine, indicated that such activity was indeed present in this tissue. To confirm that normetanephrine was formed during the incubation of norepinephrine with the various tissue preparations, several criteria mave been met. We were able to account for all of the radioactivity added to the incubation flasks and a portion of this radioactivity behaved

TABLE 3. DISTRIBUTION OF COMT ACTIVITY IN PARTICULATE FRACTIONS OBTAINED FROM ADIPOCYTE LYSATES*

Fraction incubated	Total activity	Sp. act.
	Normetanephrine formed/hr (n-moles)	Normetanephrine formed/mg protein/hr (n-moles)
Experiment 1		
Adipocytes	1·12	0·050
900 *g* Sediment (ghosts)	0·87	0·105
10,000 *g* Sediment	0·07	0·057
Experiment 2		
Adipocytes	1·23	0·085
900 *g* Sediment (ghosts)	0·70	0·078
10,000 *g* Sediment	Trace	Trace
Experiment 3†		
Adipocytes	0·46	0·019
900 *g* Sediment (ghosts) No addition	0·24	0·019
900 *g* Sediment (ghosts) + 10^{-3} M normetanephrine	0·09	0·007
10,000 *g* Sediment	0·01	0·010

* In each experiment, from 5 to 6 ml of packed adipocytes were equally divided into two portions; one was used for the preparation of adipocyte ghosts, and the other was incubated as an intact cell preparation. Experimental conditions are as described in Methods, with the exception that Krebs bicarbonate buffer was not added to the incubation medium.

† In experiment 3, 0·012 μmole *l*-norepinephrine bitartrate was added to the incubation medium. The 900 *g* sediment (ghosts) was divided into two equal fractions, one being incubated in the presence of 10^{-3} M NM and the other serving as a control.

like normetanephrine in the chromatographic isolation procedures described by Iversen *et al.*[9] This radioactive material, pooled from several experiments, behaved during polyamide TLC in a manner identical with that of authentic normetanephrine. That the radioactive material was not artifactually produced from traces of norepinephrine during the extraction and plating procedures of the chromatography was verified. Finally, when the tissue preparations were incubated with either a high concentration of normetanephrine or with pyrogallol, a significant degree of inhibition in the metabolite's formation was detected.

It is quite clear that whole pieces of adipose tissue exhibit greater COMT activity than the isolated adipocytes. This difference can probably be ascribed to the presence,

in whole tissue, of nerve endings and blood vessels which contribute to the total activity. Rodbell[1] has attributed the greater lipolytic potency of ACTH on isolated cells compared with whole tissue to the greatly enhanced diffusion of fatty acids from the cells to the incubation medium. In the case of norepinephrine, a lesser degree of metabolic inactivation via the COMT reaction may also play a role in this observed enhanced lipolytic activity in adipocytes.

Of considerable interest is the finding that the major part of the COMT activity is associated with particulate material in isolated fat cells. In other tissues (and species) it appears that the enzyme is located almost exclusively in the supernatant fraction of a 78,000 *g* centrifugation of homogenized tissue.[2] As demonstrated in Table 2, the 30,000 *g* preparations which we initially used had the COMT activity distributed in a ratio of approximately 2:1, sediment to supernatant, indicating that the major portion of activity is associated with the particulate fraction. That it was not all present in the particulate was apparent, because even after 100,000 *g* centrifugation some activity was retained by the supernatant fraction. Thus we are unable to exclude the possibility of there being two enzymes functioning to 3-*O*-methylate the catecholamines. However, after much of this work had been completed, the preparation of adipose cell "ghosts" was described by Rodbell.[6] This preparation seemed ideally suited to determine the distribution of the enzyme in this tissue. The gentle rupture of the adipose cells produces a preparation of isolated plasma membranes which sediments at low gravitational forces. The major activity of the enzyme can now be found with this fraction of the cell (see Table 3). From the enzyme patterns described by Rodbell, one can assume that there is very little of the "soluble" portion of the cell remaining with these ghosts. Hence we may assume that a very major portion of the COMT activity is located in association with particulates and probably with membranes. Considerable theoretical importance may be attached to the idea that COMT is associated with membranes when one considers the current hypothesis that injected and circulating catecholamine seems to be enzymatically handled initially and primarily by COMT[14] and secondarily by monoamine oxidase. To have the enzyme located in the membrane would add considerable credibility to the hypothesis. It would be of great interest to attempt to prepare such ghost fractions from other tissues (such as liver) to examine them for possible membrane COMT. However, the possibility remains that adipose cells may be quite different from other cells, since a difference in the sedimentation characteristics for another "soluble" enzyme was reported by Rodbell.[6] In lysates of fat cells, he found a large fraction (50 per cent) of the hexokinase activity associated with the 10,000 *g* particulate fraction.

In the light of the findings reported herein, it appears quite clear that the reasons Stock and Westermann[3] were unable to detect COMT activity in white adipose tissue were 2-fold: the use of a method for detecting COMT activity which has low sensitivity, and the use of the 30,000 *g* supernatant fraction of adipose tissue homogenate which exhibits a small fraction of the activity.

Thus three pieces of information are demonstrated, which may be considered as follows: (1) both isolated fat cells and whole adipose tissue are capable of metabolizing ^{3}H-norepinephrine to a substance similar to normetanephrine by several criteria (2) the COMT activity exhibited by whole tissue is greater than that of isolated cells, and this difference in the metabolic disposition may partially account for the potency difference observed in these two preparations (3) the major share of adipocyte COMT

activity resides in a particle-bound form, probably associated with the plasma membrane.

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REFERENCES

1. M. RODBELL, in *Handbook of Physiology*, p. 471. American Physiology Society (1965).
2. J. AXELROD and R. TOMCHICK, *J. biol. Chem.* **233**, 702 (1958).
3. K. STOCK and E. O. WESTERMANN, *J. Lipid Res.* **4**, 297 (1963).
4. G. J. TRAIGER and D. N. CALVERT, *Pharmacologist* **9**, 254 (1967).
5. J. J. LECH and D. N. CALVERT, *J. Lipid Res.* **7**, 561 (1966).
6. M. RODBELL, *J. biol. Chem.* **242**, 5744 (1967).
7. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193**, 265 (1951).
8. E. VAN HANDEL and D. B. ZILVERSMIT, *J. Lab. clin. Med.* **50**, 152 (1957).
9. L. IVERSEN, J. GLOWINSKI and J. AXELROD, *J. Pharmac. exp. Ther.* **151**, 273 (1966).
10. G. BRAY, *Analyt. Biochem.* **1**, 279 (1960).
11. I. KOPIN, J. AXELROD and E. GORDON, *J. biol. Chem.* **236**, 2109 (1961).
12. R. SEGURA CARDONA and K. SOEHRING, *Medna. exp.* **10**, 251 (1964).
13. D. O. ALLEN and B. K. LUM, *Fedn Proc.* **26**, 681 (1967).
14. I. J. KOPIN, *Pharmac. Rev.* **18**, 513 (1966).