values (mg): 0·580, 0·700, 0·670, 0·460, 0·610, 0·600; mean ± S.D. 0·603 ± 0·083]. The variations are probably due mainly to differences in the thickness of the frontal slices which may be caused, for example, by temperature variations of the frozen brain during the cutting procedure.

Special problems arise when weight determinations are to be performed on *unfixed frozen tissue* that should not reach room temperature. It is then necessary to follow a strictly standardized procedure because condensation of water vapour as well as evaporation may considerably influence the weight of tissue pieces of this size. As a rule, all weight determinations were carried out on closed microhomogenizers in order to keep the total water content of the homogenizer chamber constant. The pre-cooled homogenizer was weighed without complete temperature equilibration according to the following schedule: the homogenizer was taken from dry ice and wiped for 30 sec, then exposed to ambient temperature of 24° for 60 sec. The homogenizer was subsequently wiped for another 60 sec in order to remove remaining moisture and was then weighed. Thirty seconds later, a second weight determination was carried out, which should yield the same value. In this way, variations in the weight of a given homogenizer with tissue were reduced considerably. For three different homogenizers stored on dry ice prior to weight determination, the following mean values were obtained from six determinations on a microbalance (Mettler): no. 1: mean ± S.D. 16·958766 ± 0·00021 g ($n = 6$), no. 2: 17·012005 ± 0·00017 g ($n = 6$), no. 3: 17·198992 ± 0·000081 g ($n = 6$). When the weight of the tissue contained in the homogenizer is small (2 mg), the variations are still compartively high (5–10 per cent). Nevertheless, weight determination appears to be more reliable than reference to the individual tissue cylinder as a unit.

In conclusion, the dissection technique presented herein appears to be a practical one. It permits the excision of small pieces (0·5–1 mg) of any desired area of a diameter between 0·5 and 5 mm from rat or mouse brain with sufficient precision. The main difficulties actually arise from weight determinations, especially if frozen tissue is to be weighed. It may then be useful to employ additional reference systems. According to our recent experience with monoamine determinations in these small tissue samples,[2] protein content represents a more convenient reference system.

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## REFERENCES

1. M. SCHLUMPF, Thesis, Eidgenössische Technische Hochschule, Zürich (1973).
2. M. SCHLUMPF, W. LICHTENSTEIGER, H. LANGEMANN, P. G. WASER and F. HEFTI, *Biochem. Pharmac.* **23,** 2437 (1974).

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# Effects of methadone on steroid biosynthesis in rat adrenocortical cells

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CLINICAL studies of the effect of methadone upon hypophyseal-adrenal axis have shown that 17-hydroxysteroids are within the normal range. Most[1] but not all[2] patients on methadone maintenance respond normally to insulin hypoglycemia and exogenous adrenocorticotropic hormone (ACTH), whereas diurnal rhythm[1] and cold stress response are impaired.[3] Neither of these studies incorporated a direct measure

of ACTH. In addition, there are no studies known to us in which the effect of methadone on adrenal corticosteroidogenesis has been measured directly, although morphine has been shown to inhibit adrenal steroidogenesis.[4] It was the purpose of this study to measure the direct effect of methadone upon ACTH-stimulated adrenal corticosteroidogenesis.

Adrenals were recovered from 200 to 240 g male Sprague–Dawley rats. Cells were isolated and incubated according to the procedure described by Swallow and Sayers[5] except that the cell concn was halved as was the number of donor rats. Methadone hydrochloride (Dolophine, Lilly) was diluted in buffer and added in 50 $\mu$l. Data points represent the mean ($\pm$ S.E.) of four values obtained in two experiments. Corticosterone was measured by radioimmunoassay using antiserum to corticosterone–hemisuccinyl–bovine serum albumin. Cross-reactivity of the antiserum was: cortisol, 1·6 per cent; deoxycorticosterone, 10 per cent; testosterone, 1·6 per cent; and progesterone, 12·5 per cent. Methadone did not interfere in this assay. Whole incubates were prepared by precipitating protein with ethanol and represent extra plus intra-cellular steroid. Aldosterone was measured by radioimmunoassay.[6]

Figure 1 shows the response of the adrenal cells to ACTH in the absence of methadone. No net steroid production was obtained in the absence of ACTH. A maximal response was approximated by 500 $\mu$U/ml of ACTH for corticosterone production and > 1000 $\mu$U ACTH for aldosterone production. Half maximal response was 170 $\mu$U/ml of ACTH for corticosterone.

Two hundred $\mu$U/ml was selected as an intermediate dose of ACTH to study the dose–response of methadone. Figure 2 shows that increasing concentrations of methadone from 1 to 100 $\mu$g/ml inhibited both corticosterone and aldosterone production and that complete inhibition of corticosterone and aldosterone production was obtained at 100 $\mu$g/ml of methadone. Fifty per cent inhibition of corticosterone production occurred at 21·4 $\mu$g/ml of methadone and at 10 $\mu$g/ml for aldosterone.

Having defined that methadone does inhibit steroidogenesis with a half maximal dose of ACTH, we then elected to investigate the dose response to ACTH in the presence of a half maximally inhibitory dose of methadone (15 $\mu$g/ml). Figure 1 shows that maximal corticosterone production required 10 times the ACTH (5000 $\mu$U/ml) required in the absence of methadone. Half maximal corticosterone production was achieved at 692 $\mu$U/ml of ACTH in the presence of methadone.

Figure 1 clearly shows that methadone inhibited the production of aldosterone. The maximal aldosterone production in response to ACTH was less than 30 per cent of the production obtained in the absence of methadone. This block could be due either to a selective effect on glomerulosa at a point prior to progesterone or it could be due to an inhibition of the 18-hydroxylase. Further studies will be required to clarify the point.

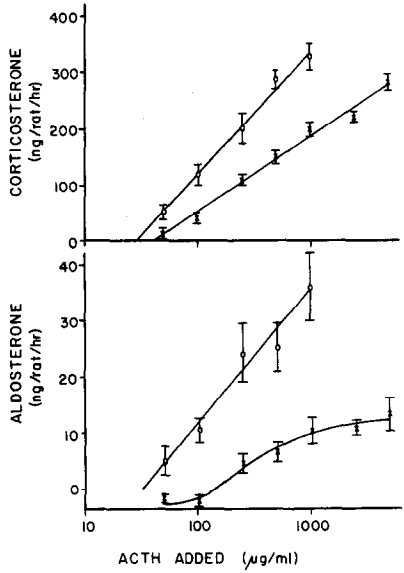


FIG. 1. Effects of methadone (15 $\mu$g/ml) on the production of corticosterone and aldosterone by rat adrenolcortical cells in response to various doses of ACTH. Open circles represent zero methadone; × represents added methadone.

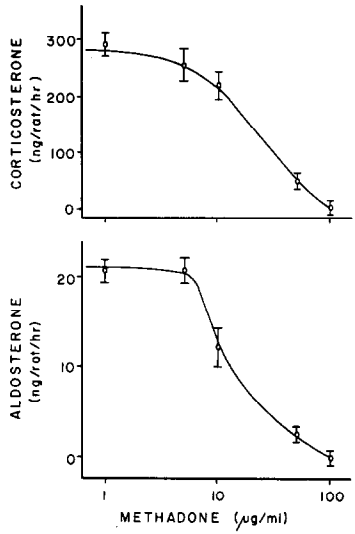


FIG. 2. Inhibition of corticosterone and aldosterone production in response to ACTH (200 μU/ml) by various doses of methadone.

The mechanism by which methadone inhibits ACTH stimulation of steroidogenesis is not clear. There is no evidence to date for an effect of morphine on the adenylate cyclase system to my knowledge. There are data which show that morphine blocks calcium transport in the muscle. Since calcium is required for a response to ACTH,[7] it is possible that a blocking of calcium transport which has been reported for morphine[8] could result in the inhibition of adrenal steroidogenesis as well. It is also possible that the inhibition may be due to competition inhibition of one or more of the enzymes involved in the conversion of cholesterol to corticosterone and aldosterone. The ability of larger doses of ACTH to overcome the effects of methadone argues against a non-specific cytotoxicity. Further investigation will be required to elucidate the mechanism of this inhibition.

The doses required to obtain inhibition *in vitro* differed from blood levels observed in addicts on a maintenance dose of methadone[9] by only about an order of magnitude. This is not a great difference in terms of the comparisons *in vitro* vs *in vivo*. Thus, while gross measures of adrenal responsiveness (17-hydroxysteroids) appear to be within the normal range *in vivo*[1] in a majority, an effect in these subjects cannot be completely excluded without directly measuring ACTH. There are no reports in the literature, to our knowledge, regarding abnormalities of salt and water metabolism in addicts on high doses of methadone. In view of the greater sensitivity of glomerular aldosterone production to methadone, a metabolic study of electrolyte, water and aldosterone metabolism in methadone users may be warranted.

It has been recently reported that mean plasma testosterone concentration was significantly decreased in male methadone patients with a non-significant increase in gonadotropins.[10] It is possible that testicular testosterone biosynthesis may be affected in the same manner as is reported herein for adrenal steroidogenesis.

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## REFERENCES

1. P. CUSHMAN, JR., B. BORDIER and J. G. HILTON, *J. clin. Endocr. Metab.* **30,** 24 (1970).
2. P. CUSHMAN, JR., *J. clin. Endocr. Metab.* **35,** 352 (1972).
3. P. F. RENAULT, C. R. SCHUSTER, R. L. HEINRICH and B. VON DER KOLK, *Clin. Pharmac. Ther.* **13,** 269 (1971).
4. T. NAKAO, K. HIRAGA, M. INABA and Y. URATA, in *Steroid Dynamics—Proceedings of Symposium on Dynamics of Steroid Hormones* (Eds. G. PINCUS, T. NAKAO and J. TAIT), p. 179. Academic Press, New York (1966).
5. R. L. SWALLOW and G. SAYERS, *Proc. Soc. exp. Biol. Med.* **131,** 1 (1969).

6. R. W. FARMER, D. H. BROWN, P. Y. HOWARD and L. F. FABRE, JR., *J. clin. Endocr. Metab.* **36,** 460 (1973).
7. M. K. BIRMINGHAM, E. KURLENTS, R. LANE, B. MUHLSTOCK and H. TRAIKOV, *Can. J. Biochem. Physiol.* **38,** 1077 (1960).
8. A. S. FAIRHURST, J. MACRI and N. HERLIHY, *Life Sci.* **10,** 1133 (1971).
9. C. E. INTURRISI and K. VEREBELY, *Clin. Pharmac. Ther.* **13,** 633 (1972).
10. F. AZIZI, A. G. VAGENAKIS, C. LONGCOPE, S. H. INGBAR and L. E. BRAVERMAN, *Steroids* **22,** 467 (1973).

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Biochemical Pharmacology, Vol. 23, pp. 2452–2454. Pergamon Press, 1974. Printed in Great Britain.

## The *in vivo* formation and turnover of *S*-adenosylmethionine from methionine in the liver of normal rats, of animals fed dimethylnitrosamine, and of partially hepatectomised animals

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*S*-ADENOSYL-L-methionine (SAM) was first shown by Cantoni[1] to be the form in which methionine acts as a methyl donor. The metabolic importance of SAM is illustrated by the fact that it is the active intermediate in more than 40 transmethylation reactions. Thus it is involved in methylation of diverse compounds of low molecular weight,[2] and also of macromolecules including myosin,[3,4] nuclear protein,[5] transfer RNA[6] and DNA.[7] The metabolism of these compounds *in vivo* is often studied by giving methionine labelled in the methyl group to intact animals, and later the extent of labelling of SAM and of the compound under investigation are determined. This approach assumes knowledge of the rate of synthesis of SAM from methionine, the specific radioactivity of SAM which is attained, and also of the time period for which the specific activity is high in the tissue under consideration. However, the rapid rate of turnover of SAM[8] has not always been appreciated; the specific activity of SAM reaches a peak within approx. 10 min of injection of methionine and then rapidly decreases. Therefore measurements made at 1 hr[9] are difficult to interpret.

During investigations of biochemical changes involved in carcinogenesis, the methylation of transfer RNA[10] and of DNA [11] was studied after injection of [$^{14}$C]methionine into animals fed a diet containing dimethylnitrosamine. The results suggested that t-RNA became more highly methylated in precancerous liver, but that the methylation of DNA increased only in proportion to the increased rate of synthesis. To determine how far these changes might be explained not by altered methylation of nucleic acids but by altered metabolism of the methyl donor, SAM, it was necessary to study the turnover of SAM during carcinogenesis. This was of special interest as there is in fact evidence for deranged l-carbon metabolism during carcinogenesis. Thus there is evidence for an increased concentration of SAM in leukaemic white cells[12] and in a neuroblastoma,[13] and for a decreased level of SAM synthetase, ATP: L-methionine adenosyl transferase EC 2.4.2.13, in a mouse hepatoma[14] and in Novikoff hepatoma.[15] The turnover of SAM was therefore studied in the liver of rats fed the carcinogen dimethylnitrosamine, under conditions used in the previous study of methylation of t-RNA and DNA.[10,11]

There is little information concerning the formation of SAM in dividing cells. This is of interest as replicating cells are a suitable system for studying how closely methylation of DNA, t-RNA and histones is geared to the synthesis of these macromolecules. The specific activity of SAM was therefore studied in regenerating liver at different times after administration of [$^{14}$C]methionine.

## MATERIALS AND METHODS

L-[Me$^{14}$C]methionine, 56·8 mCi/m-mole, was purchased from The Radiochemical Centre, Amersham, Bucks.

Female rats, 190–210 g body wt, were used.

The animals treated with labelled methionine were either normal rats, or animals which had been partially hepatectomised by the method of Higgins and Anderson.[16] Female rats were fed a diet containing 50 ppm dimethylnitrosamine from the time they were 100 g body wt, for a period of 18 weeks, by which time the body wt was approximately 200 g. Under this feeding regime rats now begin to die with liver tumors after 23 weeks instead of at 26 weeks as recorded previously.[17] At 18 weeks the gross anatomy of the liver is abnormal. Very often the two parts of the median lobe are of unequal size, one having