was adjusted to contain 2.5–3.0 $\times 10^6$ cells in a volume of 2 ml in the incubation vial. The vials were incubated for 1 h in a shaking water bath at 37 °C under 95% O_2–5% CO_2. The samples were frozen immediately after incubation and stored in a freezer until assayed for steroids.

Extraction, purification and identification of steroids were carried out exactly by a procedure described by REMBIESA et al.[1]. Radioactive counting was performed in a SL-30 Liquid Scintillation Spectrometer (Intertechnique, Paris). Radioactivity obtained was corrected for quenching by external standard method.

Results and comments. The pattern of steroids synthesized from pregnenolone-4-^{14}C and progesterone-4-^{14}C of placental cells is shown in the Table. The rat placental cells produce androgens from C_{21} steroids. 17-hydroxyprogesterone and 4-androstane-3,17-dione were obtained as the metabolites of pregnenolone and progesterone, and 3α-hydroxy-5α-pregnan-20-one and 3α,17-dihydroxy-5α-pregnan-20-one were isolated as the products of the precursors reduction. From pregnenolone, progesterone was also isolated.

Percentage conversion of radioactive precursors to various metabolites in free cell suspensions from rat placental tissue

Metabolites	Precursor	
	Pregnenolone	Progesterone
Unknown metabolite	9.7	8.3
3α,17-Dihydroxy-5α-pregnan-20-one	11.0	14.8
17-Hydroxyprogesterone	2.4	2.6
4-Androstene-3,17-dione	4.5	9.5
3α-Hydroxy-5α-androstan-17-one	—	14.5
3α-Hydroxy-5α-pregnan-20-one	22.5	33.4
Pregnenolone	45.3	—
Progesterone	4.2	14.4
5α-Pregnane-3,20-dione	1.0	2.7

The percentage conversion of labelled precursors was calculated by eluting radioactive peaks present on chromatograms (average of 4 incubations). For other details of incubation see text, and of extraction, purification and identification see the paper[1].

The comparison of the results presented here with those reported earlier[1] indicate that free cell suspensions from rat placenta reveal the pattern of steroidogenesis similar to that of placental quarters but different from that of homogenates. Placental homogenates convert progesterone only to reduced metabolites[1,5].

Our preliminary results revealed that gradient centrifugation of the free cell suspensions results in several distinct fractions. We believe that using these fractions in studies on steroid synthesis will enable us to obtain better insight into the role of tropic hormones on placental steroidogenesis.

Zusammenfassung. Methode zur Gewinnung einer Suspension isolierter Plazentazellen der Ratte mittels dreier Enzyme (Trypsin, Kollagenase, Streptodornase). Ähnlich wie bei Placentagewebeschnitten[1], wurde die Steroidenzymaktivität festgestellt.

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Institute of Pharmacology, Polish Academy of Sciences, 52 Ojcowska Street P–31-344 Kracow (Poland), 20 September 1973.

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A New Spectrophotometric Determination of Nitrazepam

It is known that compounds having active methylenic groups give rise with the quinones to the CRAVEN reaction[1–4]. We have taken interest in the action of mononuclear and binuclear quinones on several substances of toxicological interest having an active methylenic group, e.g. barbituric acid, brucine, strychnine, diazepam and nitrazepam, in order to establish either the nature of the reaction products or the analytical employment of such reactions. The reaction between barbituric acid and chloranil has been reported in a previous note[5]. We have now examined the reaction between nitrazepam and several quinones in dimethylformamide (DMF) solution in presence of ammonia, and we have found that 1,2-naphtoquinone gives the more sensitive chromatic reaction. The stoichiometry of this reaction has been established spectrophotometrically, according to the method of continuous variations of JOB[6]. On the basis of the results obtained, we can affirm that nitrazepam and 1,2-naphtoquinone give rise in solution to a molecular adduct with molar ratio 1:1 between the components. The optical density of this orange DMF solution is a linear function of nitrazepam concentration in the range $0.125 \times 10^{-4} \div 2.5 \times 10^{-4}$ moles/l. The suitability of this method of inquiry has been verified by adding variable and known amounts of nitrazepam (in the above range) to a series of known samples of the biological liquids blood-serum, saliva and urine. In the blood-serum, the quantity of nitrazepam evaluated was 95 ± 1.5% of the present amount; in the saliva the 96 ± 2% and in the urine the 94 ± 3%. Such results are better than those reported with other methods[7,8], and therefore this chromatic reaction

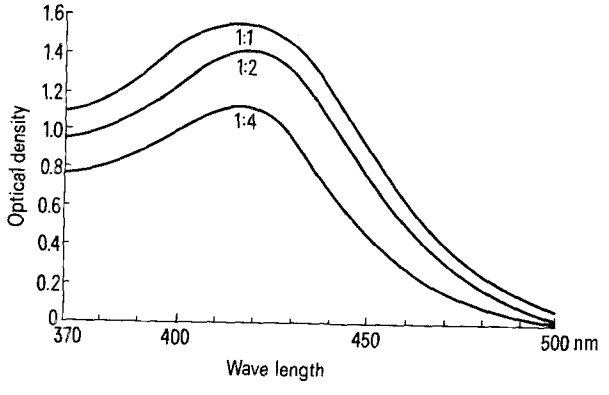

Fig. 1. Absorption spectra of ammoniacal DMF solutions containing nitrazepam and 1,2-naphtoquinone in molar ratios 1:1, 1:2, 1:4.

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may be used successfully in the spectrophotometric dosage of nitrazepam, also for the rapidity and facility of working procedures.

Experimental. Visible spectra were recorded using a Rank Precision, Uvichem H 1600 S.T. Spectrophotometer; 1-cm stoppered fused silica cells were used. Nitrazepam (F.I.S. SpA, Vicenza, Italy) and 1,2-naphtoquinone (Fluka AG, Buchs, Switzerland) were purified by crystallization from ethanol and benzene respectively. Dimethylformamide was reagent grade for spectrophotometry. Solutions of nitrazepam and of 1,2-naphtoquinone in DMF, both of concentration 5×10^{-4} *M* were prepared. The DMF solution of nitrazepam contains also the 10% by volume of ammonium hydroxide (26° Bé). Solutions of nitrazepam and of 1,2-naphtoquinone were mixed in molar ratio 1:1, 1:2 and 1:4, and the optical densities of these mixtures were measured: the absorption spectra (Figure 1) show that the curves do not cross in the explored region of the spectrum. Then, a series of mixtures was prepared by adding x ml of 5×10^{-4} *M* 1,2-naphtoquinone solution to $(1-x)$ ml of 5×10^{-4} *M* nitrazepam solution,

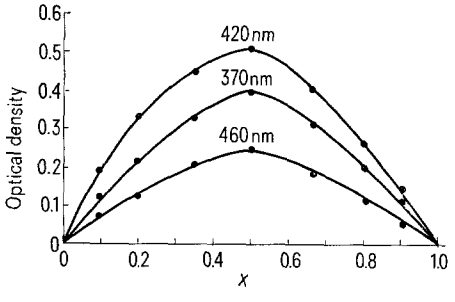

Fig. 2. Absorption spectra of mixtures of x ml of 5×10^{-4} *M* 1,2-naphtoquinone solution with $(1-x)$ ml of 5×10^{-4} *M* nitrazepam solution.

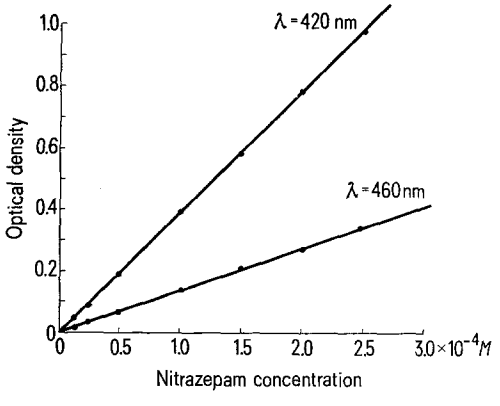

Fig. 3. Plots of optical density vs. nitrazepam concentration according to the law of Lambert-Beer.

and the optical densities of these mixtures were measured at wave lengths of 370, 420 and 460 nm (Figure 2). All wave lengths gave a definite maximum at $x = 0.5$: this is what would be expected for substances that react only in the molar ratio of one to one. In order to establish the range of nitrazepam concentration in which the optical density of the DMF solution containing the 2 components follows the law of Lambert-Beer, a series of samples was prepared by adding known and increased volumes (in the range 0.25 ÷ 5 ml) of ammoniacal DMF solution of nitrazepam to 5 ml of DMF solution of 1,2-naphtoquinone; the samples were diluted to constant volume of 10 ml with DMF and allowed to stand for 5 min before reading the optical densities. The reference sample was made by adding 1 ml of ammonium hydroxide (26° Bé) to 5 ml of DMF 5×10^{-4} *M* 1,2-naphtoquinone solution, and diluting to 10 ml. Figure 3 shows the linear correlation between optical density and nitrazepam concentration in the range $0.125 \times 10^{-4} \div 2.5 \times 10^{-4}$ moles/l, corresponding to a content of nitrazepam between 3.5 and 70 µg/ml. The reliability of this colorimetric determination in toxicological chemistry was verified on samples of blood, saliva and urine. To this purpose, one-ml samples of biological liquid of known and variable amounts (in the above range) of nitrazepam (DMF solution 5×10^{-4} *M*) were added. Each one of these samples was treated with 2 ml of 30% aqueous solution of trichloroacetic acid; after stirring for a few min, the precipitate was filtered and then washed with 1 additional ml of trichloroacetic acid. The collected filtrate was concentrated (without loss) by moderate heating and, after cooling, mixed with 1 ml of ammonium hydroxide (26° Bé) and 1 ml of 2.5×10^{-3} *M* 1,2-naphtoquinone DMF solution. The volume was made up to 10 ml and after 5 min the extinction of the colored solution was measured at 420 nm. The reference sample was the same as mentioned above. The quantity of nitrazepam evaluated in the blood-serum was 95 ± 1.5% of the added amount; in saliva 96 ± 2% and in urine 94 ± 3%. All the manipulations require a time of 20 min.

Riassunto. È stata studiata per via spettrofotometrica la reazione cromatica tra nitrazepam e 1,2-naftochinone in dimetilformammide (DMF) in presenza di ammoniaca. È stato stabilito che in soluzione si forma un addotto molecolare dei due reagenti in rapporto molare 1:1. Tale reazione viene proposta come metodo di determinazione colorimetrica del nitrazepam in liquidi biologici quali sangue, saliva ed urina.

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