Quantitative estimation of corticosteroids by means of ^{14}C-blue tetrazolium

In 1941 KUHN AND JERCHEL[1] reported that tetrazolium derivatives can be used as indicators of biological reducing processes. MADER AND BUCK[2] and CHEN *et al.*[3] have since described the reduction of blue tetrazolium (BT) to coloured formazans by corticosteroids. NOWACZYNSKI *et al.*[4] found that BT can be used for the quantitative colorimetric determination of corticosteroids with a sensitivity of approximately 0.5 μg. WEISZ *et al.*[5] were able to increase the sensitivity of the method by using ^{14}C-BT and measuring the radioactivity of the ^{14}C-formazans. Due to differences in polarity, excess BT can be separated quantitatively from formazan. The present paper presents technical data on the determination of aldosterone and corticosterone in biological extracts by means of ^{14}C-BT.

Materials

5,5'-^{14}C-Blue tetrazolium (3,3'-dianisole-bis[4,4'-(3,5-diphenyl)]tetrazolium chloride), Reanal, Budapest. Specific activity: 1.148 mC/mM.

1-2-^{3}H-*d*-Aldosterone (pregn-4-ene-11,21-diol-3,20-dion-18-al), New England Nuclear Corp., Boston. Specific activity: 20 C/mM. 1-2-^{3}H-Corticosterone (pregn-4-ene-11,21-diol-3,20-dione), New England Nuclear Corp., Boston. Specific activity: 500 mC/mM. Cortisol (pregn-4-ene-11,17,21-triol-3,20-dione), Schering, Berlin. Corticosterone, Schering, Berlin. *d*-Aldosterone, CIBA, Basel. Each compound of chromatographic purity.

Dichloromethane pa., Merck, Darmstadt, redistilled before use. Ethanol p.a., Merck, Darmstadt, column redistilled before use.

Methods

Isolation of steroids for quantitative analysis. 120–180 g male Sprague-Dawley rats were decapitated, their adrenals removed, quartered and incubated as separate samples (50–120 mg adrenals of 1–3 rats) in 10 ml Krebs-Ringer-bicarbonate–glucose solution under a stream of 95 % O_2 and 5 % CO_2 at 37° for 240 min. The corticosteroids were extracted from the incubation medium and isolated by means of paper chromatography (Fig. 1, top). After separation the steroids were eluted from the (IB/MW)* chromatograms with 0.5 ml ethanol, 2 ml dichloromethane and 2 ml dichloromethane successively. The solvent was removed in a rotatary vacuum evaporator at 37°. The dry residue was dissolved in dichloromethane and cooled to 0° in ice. Losses of steroid material during extraction and isolation procedures were calculated from the recovery of ^{3}H-labelled steroid standards (20000–50000 imp./min) added to the incubation medium prior to extraction.

Preparation of filter paper for ^{14}C-BT reaction. Whatman No. 1 filter paper was purified by continuous descending chromatography with 10 % NaOH solution. After 48 h the solution was replaced by methanol and the sheet chromatographed for another 12 h. To remove the last traces of NaOH the paper was repeatedly washed with methanol and finally dried at room temperature.

Lambda pipettes (10 or 20 λ) were used to apply equal volumes of the samples

* IB/MW = Isooctane–benzene–methanol–water (26:13:16:4).

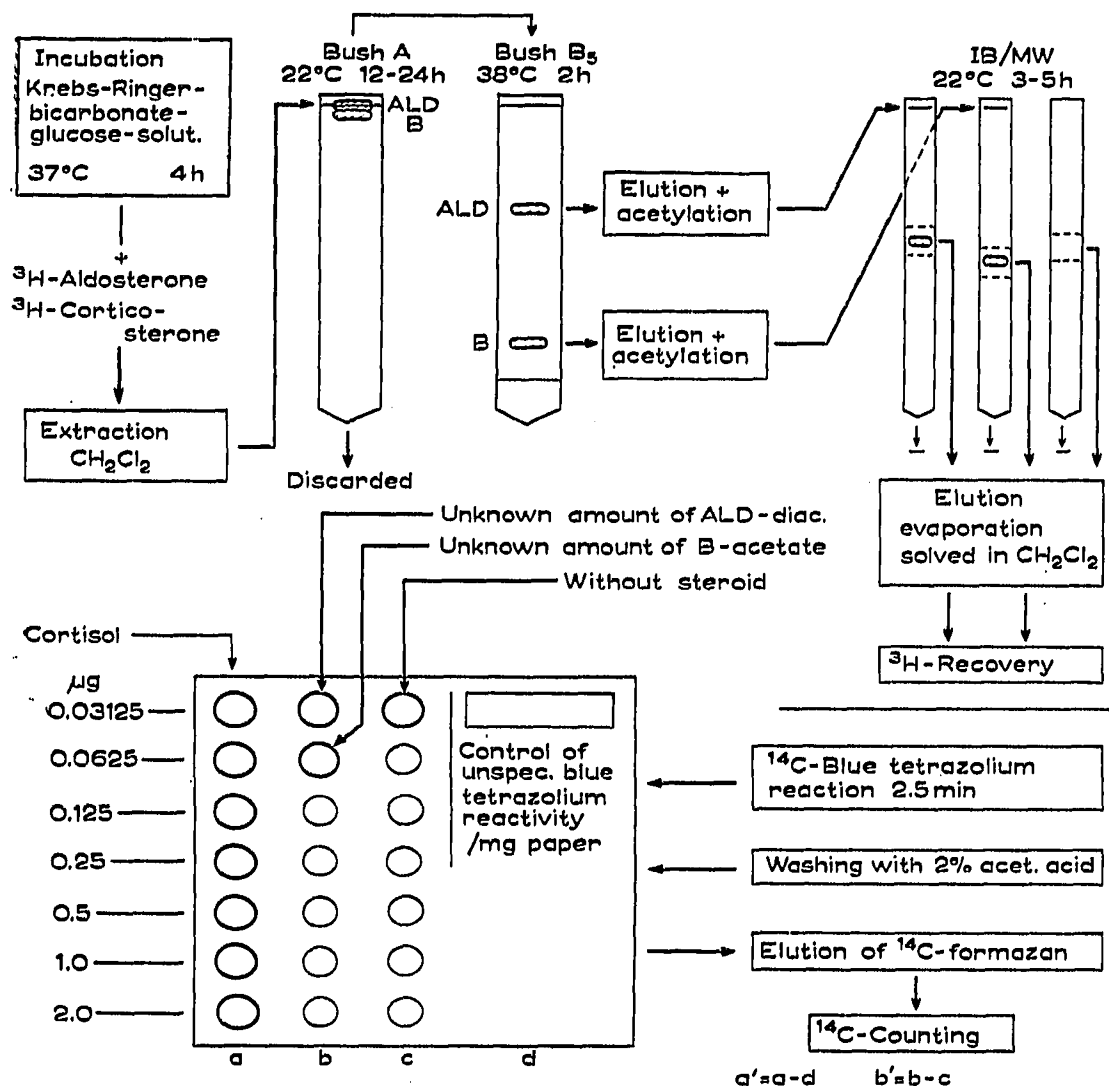

Fig. 1. Incubation and isolation procedures and ^{14}C-BT reaction on filter paper. Chromatographic systems: Bush A = *n*-heptane–methanol–water (100:80:20); Bush B_5 = benzene–methanol–water (100:55:45); IB/MW = isooctane–benzene–methanol–water (26:13:16:4).

to the prepared filter paper for the BT reaction as illustrated in Fig. 1. The following were examined:

(a) calibrated amounts of standard cortisol dissolved in ethanol;

(b) spots of dichloromethane solutions, containing unknown amounts of isolated corticosteroids;

(c) spots of dichloromethane solution obtained after chromatography in the final system (IB/MW) without steroid;

(d) an area for unspecific BT-reactivity of filter paper calculated per mg paper weight.

After application of the samples the paper was sprayed with *n*-heptane and kept for 12 h in a heptane atmosphere.

BT-reaction. 6–10 mg of ^{14}C-BT was dissolved in 1 ml ethanol, diluted with 20 ml 10 % NaOH, filtered and poured over the prepared filter paper. After 2.5 min

the BT–formazan transformation was stopped by replacing the alkaline ^{14}C-BT solution with 60 ml 2% acetic acid. To remove excess ^{14}C-BT and corticosteroids the paper was left in the bath of 2% acetic acid for 20 to 25 min, changing the solution every 5 min. The paper was then dried at 21°.

Circular areas, slightly greater than the formazan spots, were cut from the paper and each piece was weighed. The ^{14}C-formazan from each paper sample was eluted with ethylacetate–methanol (7:3) for 3 h. The solvent was removed under vacuum and the dry residue dissolved in scintillation fluid (LOMMER AND WOLFF[6]). The ^{14}C-formazan radioactivity of the samples was measured in a Packard Tri-Carb spectrometer.

Calculation. Radioactivity values (a') for a calibration curve (Fig. 2) were calculated as:

$a' = a - d$ (counts/100 min)

where:

a = formazan radioactivity eluted from the corresponding cortisol spots (Fig. 1, bottom),

d = background formazan radioactivity eluted from paper blanks of corresponding weight.

The formazan radioactivity (b') produced by unknown amounts of aldosterone or corticosterone was calculated from:

$b' = b - c$ (counts/100 min)

where:

b = formazan radioactivity eluted from the corticosterone-acetate and aldosterone-diacetate spots,

c = formazan radioactivity eluted from corresponding spots of the IB/MW-blank.

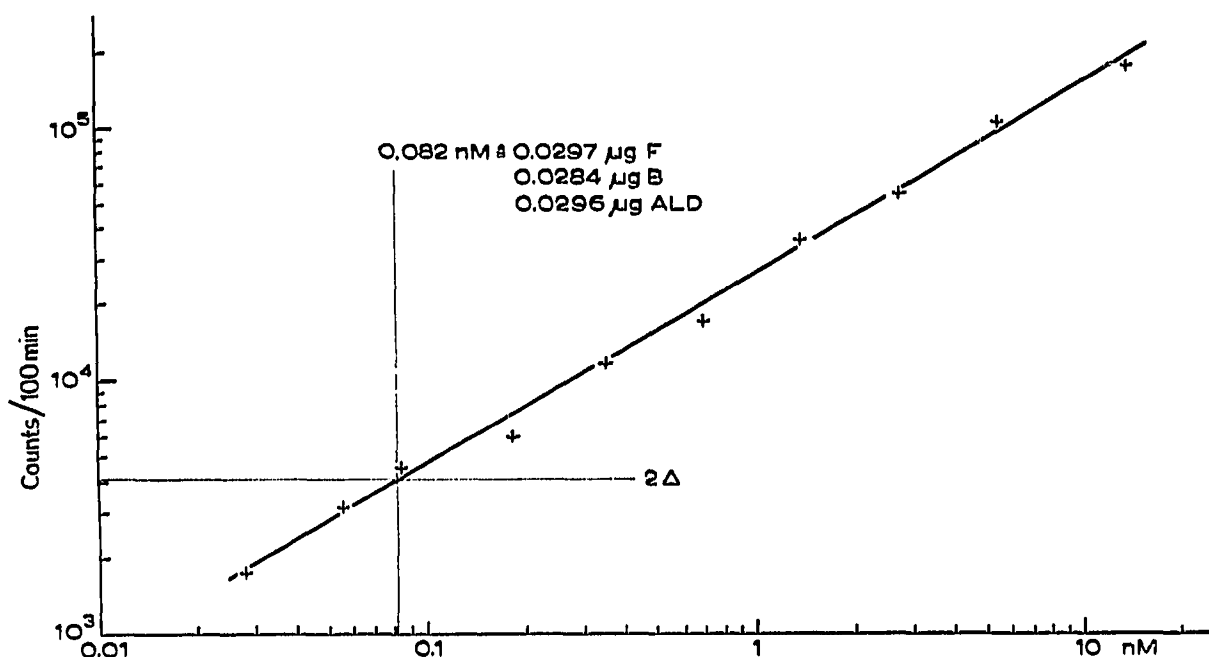

Fig. 2. Calibration curve.

Results

Sensitivity. Fig. 2 shows a calibration curve produced with cortisol (a' values). In a range of 0.01–5.0 μg (= 0.0277–13.8294 nM) the reaction is represented by a linear function The sensitivity of the technique is limited by the error $\pm \Delta$,:

$$\Delta = \sqrt{(SD_c^2 + SD_{BG}^2 + SD_{TRI\text{-}CARB}^2)} \text{ (counts/100 min)}$$

where:

SD_c = standard deviation of the c values
SD_{BG} = standard deviation of the ^{14}C-background
$SD_{TRI\text{-}CARB}$ = standard deviation of ^{14}C-counting

In the example in Fig. 2, Δ equals $\pm$ 2050 counts/100 min ($n_c = 10$). Reliable values can be expected when b' exceeds $2\,\Delta$ = 4100 counts/100 min. The sensitivity of corticosteroid determinations in biological extracts therefore approximates 0.03 μg.

Precision. To determine the precision of the assay, two series of duplicate determinations of corticosteroids were carried out in extracts obtained from incubation media of slices of rat adrenals (Tables I and II). In the first series (Table I) the results of duplicate determinations of identical samples by (a) the ^{14}C-BT-method and (b) the conventional BT reaction according to the technique of NOWACZYNSKI *et al.*[4] were compared. In the second series (Table II) corticosterone samples were estimated twice by the ^{14}C-BT method. Deviations from the mean values as derived from the individual results of both series are considered. The mean deviations of the two series of seven estimations were 5.1 ± 1.1 (SEM) % and 6.8 ± 1.9 (SEM) % for the labelled–unlabelled BT-assay and the ^{14}C-BT-method alone, respectively.

Estimation of aldosterone and corticosterone produced by the adrenals of normal and bilaterally nephrectomized rats. Aldosterone and corticosterone production was determined in the incubation medium of normal and bilaterally nephrectomized rats (Table III). The results demonstrate that the ^{14}C-BT method is suitable for the quantitative evaluation of micro amounts of corticosteroids. The corticosterone production was significantly decreased in adrenals of nephrectomized rats, while there was no alteration in aldosterone biosynthesis (see STEINACKER *et al.*[7]).

TABLE I

DUPLICATE ESTIMATIONS OF CORTICOSTERONE FROM INCUBATES USING ^{14}C-BT AND BT

Corticosterone (μg)		*Mean in μg*	*Δ in μg*	*Δ as % of mean*
^{14}C-BT	*BT*			
0.75	0.90	0.83	0.07	8
1.32	1.20	1.26	0.06	5
1.26	1.20	1.23	0.03	2
0.72	0.85	0.79	0.06	8
3.80	3.60	3.70	0.10	3
4.58	4.45	4.52	0.07	2
8.20	7.00	7.60	0.60	8
				Mean Δ = 5.1 %
				SEM = ± 1.1 %

TABLE II

DOUBLE ESTIMATION OF CORTICOSTERONE FROM INCUBATES USING ^{14}C-BT

Corticosterone (μg)		Mean in μg	Δ in μg	Δ as % of mean
^{11}C-BT	^{14}C-BT			
0.108	0.120	0.114	0.06	5
0.192	0.195	0.196	0.002	2
0.210	0.250	0.230	0.020	9
0.200	0.275	0.238	0.038	16
0.890	0.990	0.940	0.050	5
1.720	2.080	1.900	0.180	9
2.400	2.500	2.450	0.050	2
				Mean Δ = 6.8%
				SEM = ± 1.9%

TABLE III

In vitro ALDOSTERONE AND CORTICOSTERONE PRODUCTION OF RAT ADRENALS EVALUATED WITH ^{14}C-BT

	Aldosterone production (μg/h/100 mg)		Corticosterone production (μg/h/100 mg)	
	Control	Nephrectomized	Control	Nephrectomized
	1.05	1.39	4.24	0.72
	1.66	0.30	2.94	0.96
	0.93	0.60	5.37	2.95
	0.33	1.54	7.56	0.66
	0.78	0.20	3.66	0.65
	1.33	0.25	4.50	0.57
	0.26		4.99	1.27
	0.77		2.68	1.90
	1.61		3.89	
	0.12		5.78	
			7.99	
			5.21	
			7.43	
			2.27	
			1.90	
			2.27	
Mean	0.77	0.71	4.54	0.83
SEM	0.17	±0.21	±0.49	±0.29
			$p < 0.001$	

Discussion

Corticosterone and aldosterone were estimated after acetylation using a cortisol calibration curve. The BT-reaction gives the same molar extinction coefficients with cortisol, corticosterone, aldosterone, cortisol acetate, corticosterone acetate and aldosterone diacetate, as demonstrated with unlabelled BT (Fig. 3). Results in Table II show that both techniques, using ^{14}C-BT or unlabelled BT, are directly comparable. The ^{14}C-BT reaction was carried out on filter paper to allow quantita-

tive separation of excess BT from formazan by aqueous (2 % acetic acid) elution. The unspecific ^{14}C-BT reaction of the filter paper could be reduced considerably by washing with NaOH and methanol[8]. Exposure of the paper to *n*-heptane enhanced the intensity of the corticosteroid–BT reaction[9].

The sensitivity of the ^{14}C-BT method is less than that of the double isotope derivative techniques described by KLIMAN AND PETERSON[10] and BOJESEN AND DEGN[11], but about ten times higher than that of techniques using unlabelled BT (compare Fig. 1 and Fig. 3).

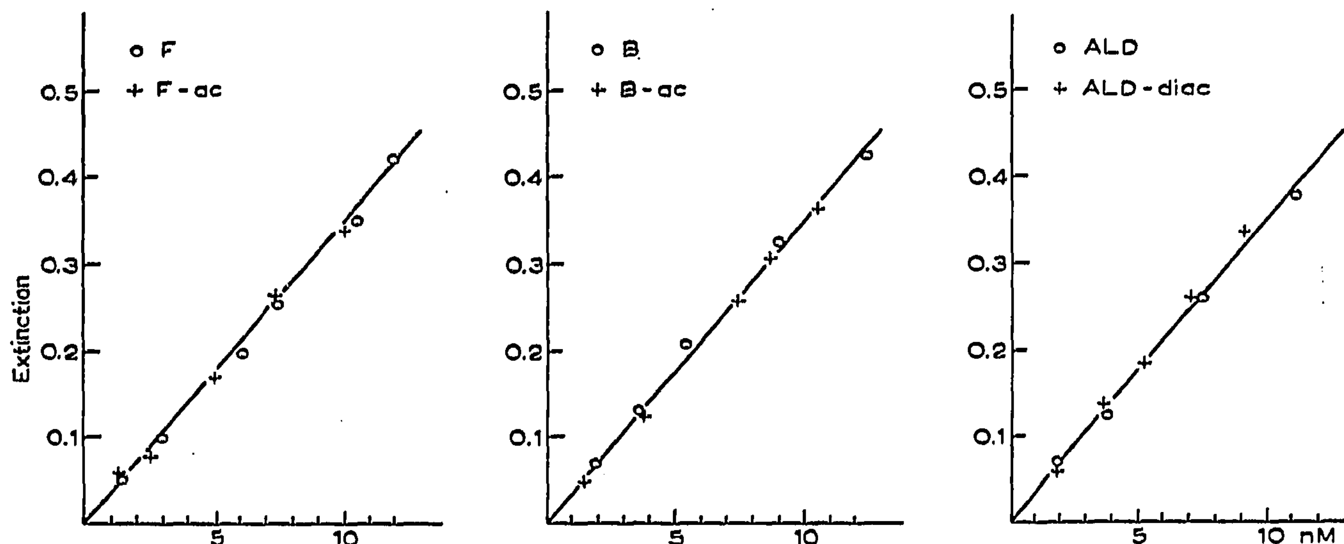

Fig. 3. BT (unlabelled) reactions of cortisol (F), corticosterone (B) and aldosterone (ALD) and their acetate derivatives.

The method described permits the quantitative determination of minimal amounts of corticosteroids in biological material and may be useful for routine steroid analysis in the clinical field.

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Some technical improvements in the paper chromatography of keto acid 2,4-dinitrophenylhydrazones

Alpha keto acids are commonly studied in biological samples, as their 2,4-dinitrophenylhydrazones[1–6] (DNP-hydrazones). In the usual extraction procedures, DNP-hydrazones of keto acids are extracted into an organic solvent followed by re-extraction into an alkaline aqueous layer. After acidification of this aqueous layer they are again taken up into an organic solvent. The last step is accompanied by loss of some of the DNP-hydrazones[3] and it has been dispensed with in certain modifications[1,3]. In these modified methods, however, one has to spot 300–500 μl of an aqueous solution, which is rather tiresome. In the method to be described in this paper DNP-hydrazones of keto acids are taken up from sodium carbonate solution without acidification into small amounts of a triethylamine–pyridine mixture (2:1). The final volume obtained in this way is comparable to the one obtained in the modifications referred to above, but it is much easier to apply.

A solvent is also described which separates the DNP-hydrazones of common keto acids in about two hours, as compact spots, almost irrespective of the area over which the sample is spotted.

Experimental

DNP-hydrazones of keto acids are prepared according to McARDLE[1].

The DNP-hydrazones are first taken up into a mixture of chloroform–ethyl alcohol (4:1) and subsequently into 1 *N* sodium carbonate solution[2]. Two extractions with 3 ml and 2 ml sodium carbonate solution, respectively, are made to obtain the DNP-hydrazones in 5 ml of the solution, corresponding to 2 ml urine or 5 ml blood.

The sodium carbonate solution, taken in a 10.5 × 1.4 cm stoppered tube, is cooled down to 0–4° and sufficient sodium carbonate is added, to obtain a saturated solution at room temperature. At 35° about 1.5 g may be needed for the purpose.

After the tube attains the room temperature DNP-hydrazones are extracted into a triethylamine–pyridine mixture (2:1). First two extractions are made, each with 0.5 ml of the mixture and subsequently two or more, each with 0.25 ml. For every extraction vigorous shaking is necessary. The layers separate out without any difficulty. In the final extraction the upper layer should be colourless.

100–500 μl of the triethylamine–pyridine extract is spotted for chromatography.