Journal of Steroid Biochemistry, 1972, Vol. 3, pp. 349–356. Pergamon Press. Printed in Great Britain

PROBLEMS ARISING FROM BLANKS IN PROTEIN-BINDING ANALYSIS

M. A. PIZARRO and J. KOLANOWSKI
Endocrine Unit, Departments of Physiology, Medicine and Medical Biochemistry, Dekenstraat 6, Université Catholique de Louvain, Louvain, Belgium

SUMMARY

"Blank" in protein-binding analysis is known to represent an entity that cannot be merely subtracted from experimental values.

Illustrations of this troublesome aspect of protein-binding analysis are provided and the precautions making it possible to eliminate "blank" in the case of corticosteroid and progesterone assays are detailed.

To this end, careful control of temperature at the binding step, treatment of glassware at 500°C for 60 min, or use of Sephadex LH-20 proved valuable in our laboratory.

The problem of "negative blank" is alluded to. It was reasoned that it only represents a particular aspect of the same problem. In other words, when there is a "blank", it could be positive or negative, depending upon several factors, only part of which are individualized.

It is suggested that in protein-binding analysis, "blank" is a complex phenomenon resulting from alterations of the protein-steroid relationship.

INTRODUCTION

TODAY, most steroid assays relying on protein binding include a chromatography, in order to achieve specificity. This step often results in appearance of some interference, generally called "the blank".

Our first attempts to correct for "blank" consisted in subtracting it from all values but we soon realized that this was a wrong approach. We summarize here the reasons which lead us to think that "blank" correction is not a mere matter of arithmetic. In addition, data assumed to be useful to avoid this "blank" problem are included.

EXPERIMENTAL

Basic conditions adopted for corticosteroid and progesterone assays by protein binding are summarized in Table 1. They involve thin layer chromatography (t.l.c.) on glass silica-coated plates purchased from Merck.

For cortisol, corticosterone and 11-deoxycortisol, glass or plastic disposable material can be employed. Corticosteroid binding globulin (CBG) was obtained from plasma contributed by healthy individuals; it was diluted with phosphate buffer to a final concentration of 3 or 0·5% (v/v); 100 ml of solution contained about 4 μCi of the appropriate tritiated steroid.

When progesterone is to be measured, only glass material should be used. CBG was provided in this case by healthy women taking 100 μg mestranol daily for at least 2 months. Their plasma was diluted to a variable extent so as to obtain a 50% displacement of tritiated corticosterone with 2 ng of progesterone.

This contribution actually deals with results obtained with "sample blanks," by which we mean samples containing only about 1000 c.p.m. of the appropriate

Table 1. Steroid-protein binding assay: Basic conditions

Steroid	Tubes	Internal standard	Protein-solution	Adsorbant	Incubation time at 1 ± 0·2°C
Cortisol (F)	G/P	^{3}H-F	CBG-^{3}H-F (3% human plasma in Phosphate buffer; v/v)	Dextran-coated charcoal in phosphate buffer	60 min to 72 hr
Corticosterone (B)	G/P	^{3}H-B	CBG-^{3}H-B (0·5% human plasma in Phosphate buffer; v/v)		
11-Deoxycortisol (S)	G/P	^{3}H-S	CBG-^{3}H-B (0·5% human plasma in Phosphate buffer; v/v)	(0·15%, 1·5% w/v)	
Progesterone (P)	G	^{3}H-P	CBG-^{3}H-B (0·07-0·14% estrogen-treated human female plasma in water; v/v)	Florisil	15–90 min

Phosphate buffer: 0·02 M, pH 6·9, G = Glass, P = Plastic.

Specific Activities of Internal Standards: ^{3}H-F: 55 Ci/mmol, ^{3}H-S: 35 Ci/mmol, ^{3}H-B: 50 Ci/mmol, ^{3}H-P: 33·8 Ci/mmol.

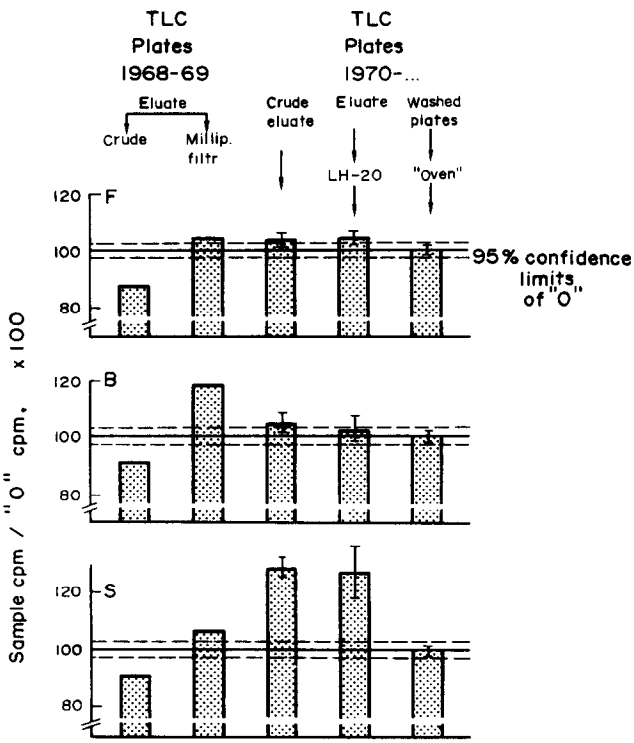

Fig. 1. Elimination of "blank" in protein binding assay. Solid line represents the zero ("0") level, with broken lines indicating 95% confidence limits. Vertical bars stand for means. For details concerning manipulation, see text.

tritiated steroid, i.e. the amount of radioactivity added as internal standard to biological samples. They were submitted to chromatography and assayed as such or after manipulation as indicated in Fig. 1.

In the "zero" tube, no steroid whatsoever was introduced; the radioactivity in the supernatant of the "zero" tube was taken as 100%, and radioactivity readings obtained for actual samples were compared to it.

RESULTS AND DISCUSSION

Pertinent data are presented in Fig. 1. Mean values, represented by bars, are generally based on at least 8 "blank sample" determinations, obtained during different runs. Values located above the reference (or "zero") line were considered as "negative blanks", since radioactivity counting in these cases was *greater* than the counts obtained for the "zero" tube. "Positive blanks" were obtained in the opposite case. When the mean and its S.D. lay within the 95% confidence limits of the "zero" tube, "blank" was considered non-existent.

Only a few representative situations are illustrated for each steroid.

Before 1970, Merck plates gave a "positive blank" that became "negative" after filtration of eluate on 0·65μ Millipore filters. This was true for all steroids tested. Afterwards, and probably on account of modifications in binder introduced by the manufacturer, "blanks", except for 11-deoxycortisol, were closer to zero but they still were "negative". This situation could not be improved by processing the eluate through Sephadex LH-20. Aqueous-organic partition likewise failed to modify these findings.

On the other hand, careful washing of t.l.c. plates and treatment of all glassware in an electric oven at 500°C for at least 60 min resulted in elimination of "blanks", *provided* strict control of temperature at the binding step was exerted.

Similar experiments were conducted for progesterone (Fig. 2). Here, LH-20 appeared essential to improve "blanks"[1]. Treatment of glassware at 500°C by itself, although useful, was insufficient to eliminate all interference. The best results were obtained by a combination of both treatments.

A major difficulty when "blank" is not eliminated arises from the fact that

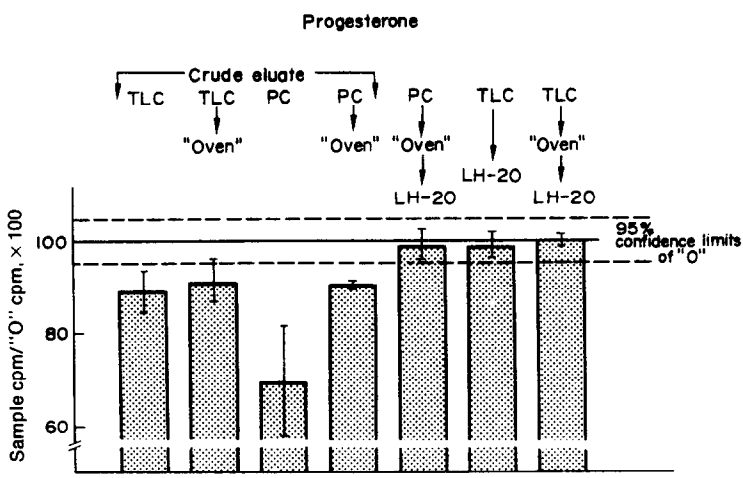

Fig. 2. Elimination of "blank" in protein binding assay. PC = paper chromatography. Other symbols as in Fig. 1. For details concerning manipulation, see text.

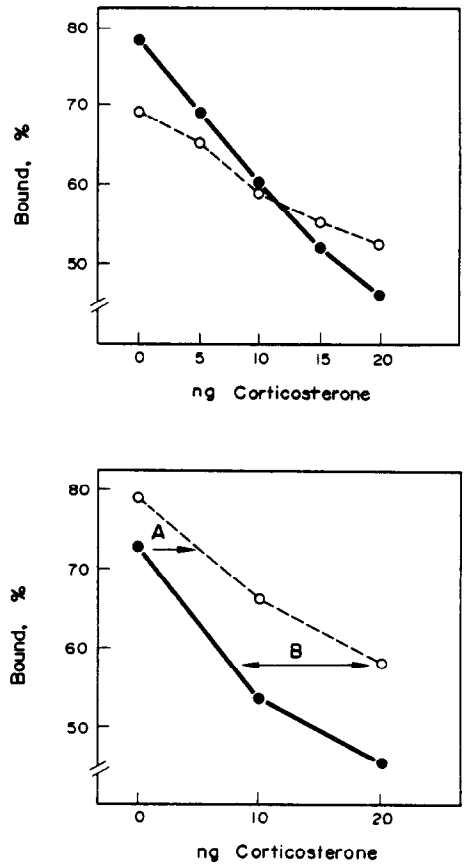

Fig. 3. Nonuniformity of blank at different concentrations of steroid. Solid line: normal standard curve. Dotted line: standard curve obtained after addition of t.l.c. eluate to different amounts of corticosterone (upper portion) or of the same eluate previously processed through Millipore filters (lower portion).

"blanks" are not necessarily uniform over the range of standard curves, as shown in Fig. 3. Per cent of remaining corticosterone bound is plotted against steroid doses in an attempt to illustrate the magnitude of changes. In the case illustrated in the upper part, despite a definite "positive blank" by criteria discussed above, only samples containing about 11 ng, at which point both curves intersect, would be reliably measured; smaller amounts would be overestimated while samples containing more than 11 ng would be underestimated if they are measured against the normal standard curve.

In the lower part of Fig. 3 a different situation is presented: all samples would be underestimated here, but the error is clearly not a constant one over the standard curve: compare B and A.

From these two examples it is obvious that subtracting (for "positive blanks") or adding (for "negative blanks") a constant, would result in erroneous values. In other words, it is not valid to correct sample figures for "blank" if the latter is not uniform for the whole range of measurements.

Even in the case of "non-existent blank" as defined, a difference could exist between both curves. In the case illustrated in Fig. 4, the error will vary, as

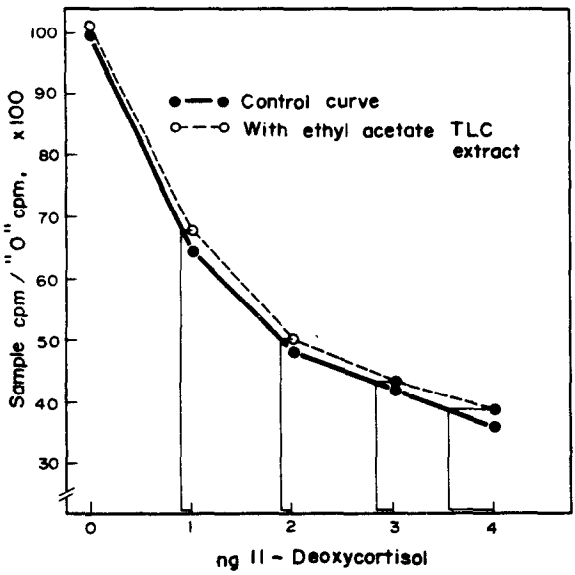

Fig. 4. Nonuniformity of blank at different concentrations of steroid. Projection on the abscissa indicates that the error varies for different amounts of steroid.

indicated by projection of the shift on the abscissa, whereas a "sample blank" is not statistically different from zero.

Similar observations have been made in the case of triiodothyronin measurements[2]. Thus, it is our belief that resorting to water for example, for blank correction, would never give adequate information concerning such shifts.

De Souza *et al.*[3] have reported a method for compensation of interfering factors ("MIF") arising especially from the use of t.l.c. plates. However, we found (cf. Figs. 3 and 4) that the contributions of "MIF" and steroid in the assay were not necessarily additive, which compromises the interest of this kind of correction.

These data suggest that several mechanisms could play a role in "blank" phenomenon.

Tentatively, the following hypotheses could be raised: (1) The unknown factor(s) could modify the tertiary structure of the binding protein, with changes in the association constants as a result[4]. If this is the case, "positive" or "negative" "blanks" would appear depending upon the direction of the shift. Also, the slope of the curve could be modified or the rate of dissociation of protein-tracer complex could be affected, enhancing in some way the binding strength of tracer to its specific protein. The latter phenomenon would become very important when bound and unbound material are separated before the reaction has come to equilibrium; chiefly, when "stripped" plasma subsequently saturated with tracer is used as source of CBG.

(2) The unknown(s) could be a molecule exhibiting some affinity for the steroid; consequently, fewer steroid molecules would be available to displace tracer from the binding protein.

(3) The unknown(s) on the other hand could compete with the steroid for the same site on the binding protein; "blank" would thus be a function of several variables, e.g. relative amounts, affinity constants, rates of equilibrium at a given temperature for the system steroid(s): unknown(s): protein(s).

At present, we don't have clearcut experimental evidence either for or against these possibilities. Most probably, "blank" represents a complex phenomenon resulting from any combination of these alterations of the "normal" interaction between protein(s) and steroid(s). Additional factors might come into play, such as interference arising from lipids or heavy metal ions[4]. Pending a better understanding of mechanisms governing these interactions, it seems to us that a practical approach would be to devote more efforts to reducing, as much as possible, interferences contributed by chromatography so as to eliminate, hopefully, *any* shift from the standard curve.

In our laboratory this was achieved for cortisol, corticosterone and 11-deoxycortisol when plates were thoroughly washed with organic solvents (once with ethyl acetate, once with chloroform/methanol 1 : 1 and once with chloroform/acetone 8·5 : 1·5), glass material was treated for at least 60 min at 500°C, and temperature was rigorously controlled at the binding step.

For progesterone analysis, for which all glass material is submitted to the same procedure, treatment of the eluate on a mini-column of Sephadex LH-20 further reduces "blank" values, thereby improving precision of the assay especially at low levels of the hormone[1].

Figure 5 is meant to illustrate the reliability of the procedures adopted: for all 4 steroids, the slope of the regression line was very close to 1, with correlation coefficients better than 0·9; significantly, the regression line describing the data can be extrapolated to the intersections of the reference axes. Furthermore, coefficients of variation were uniformly less than 10%.

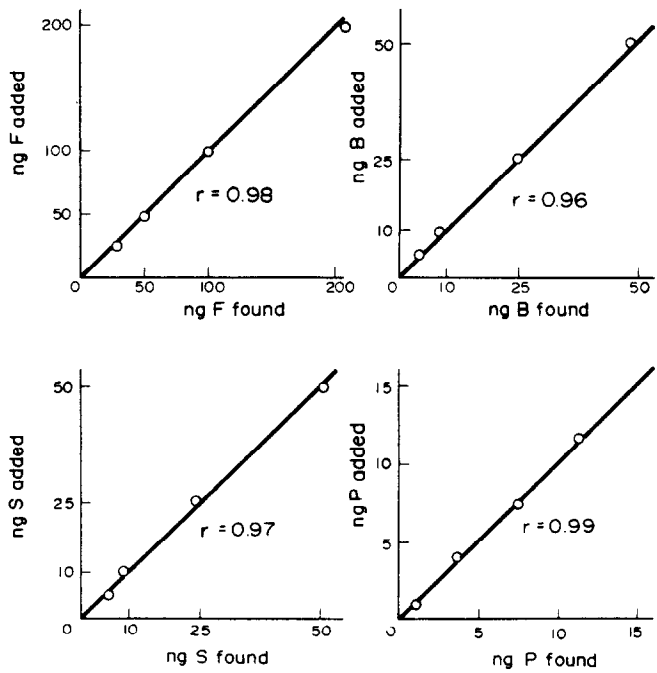

Fig. 5. Recovery of different steroids after elimination of "blank". Each point represents the mean of at least 10 determinations carried out in several runs. Cortisol, corticosterone and 11-deoxycortisol were assayed from a given sample. F = cortisol, B = corticosterone, S = 11-deoxycortisol, P = progesterone.

CONCLUSIONS

In sum, it is thought that "blank" is the consequence of multiple factors that modify the parameters governing the protein(s)-steroid interactions. It is not necessarily steady for a given protein-binding assay; thus its variations thwart figures in an unpredictable way, even when samples are processed in a given run. Therefore, if a "blank" is tolerated, no arithmetical correction for it can be applied unless "blank" be proved uniform and this at different concentrations of hormone. Better yet, "blank" should be eliminated altogether.

The appropriate conditions should be established for each steroid individually. Only when these conditions are met, can multiple hormone assays be carried out.

REFERENCES

1. M. A. Pizarro and F. Huez: *J. steroid Biochem.* **2**, (1971) 321.
2. R. P. Ekins: In *Steroid assay by protein binding* (Edited by E. Diczfalusy), 2nd Karolinska Symposium on Research Methods in Reproductive Endocrinology, Geneva (1970) (*Acta Endocr. (Kbh.)* Suppl. 147) p. 271.
3. M. L. A. de Souza, H. O. Williamson, L. O. Moody and E. Diczfalusy: In *Steroid assay by protein binding* (Edited by E. Diczfalusy), 2nd Karolinska Symposium on Research Methods in Reproductive Endocrinology, Geneva (1970) (*Acta Endocr. (Kbh.)* Suppl. 147) p. 171.
4. U. Westphal: In *Steroid-protein Interactions, Monographs on endocrinology* (Edited by F. Gross, A. Labhart, T. Mann, L. Samuels and J. Zander). Vol. 4, Springer-Verlag, Berlin (1971) Chapters XII and XIII.

DISCUSSION

Munck: Has anyone gone into the chemistry of blanks? What are these substances? Are they substances?

Pizarro: I cannot answer the question of what kind of substance gives a blank. The phenomenon is probably complex and may imply modification of the spatial arrangement of protein by many substances, rather than by one or two. We think that this was the case with our negative blanks; probably, negative or positive blanks just represent a different point of equilibrium between macromolecules and these unknown substances.

Munck: Are these substances common for different steroid analyses? Do they have the same influence on analyses of different steroids?

Pizarro: Well it could be different. There is no relation between, let me say, the amount of blank substances and the final result. If you put exactly the same volume of blank extract in a radioimmunoassay for aldosterone, for example, you get a blank that is completely different in terms of mass from the blank shown here for Substance S (11-deoxycortisol), but in per cent it's about the same thing.

Haukkamaa: Do you have any explanation for the negative blanks, because last year we also had so-called negative blanks, especially with pure distilled solvents, and the greater solvent volume evaporated, the higher was the negative blank.

Pizarro: When there is a blank it could be positive or negative. I think that a negative blank is just an artifact. Probably when you are using the very purified solvents there may be a different substance that can modify the disassociation rate or rates of the equilibrium between protein and the ligand. In this case, if you are not working at equilibrium radioactivity counting of the sample blanks or of actual samples could be greater than the zero count. When one works at equilibrium, this negative blank should disappear.

Exley: Using our competetive protein binding method for oestrogens we found that the presence of oxidizing material in our solvents may bear some relation to

the amount of the blank. Poor quality ethyl acetate, which contained peroxides, gave high blanks, but highly distilled ethyl acetate gave low blanks. We checked ether as well which is notorious for peroxides and the blank seemed to go up. So obviously it could be oxidizing material in the solvents which often caused blanks.

Piyasena: With regard to this problem of negative blanks, during the measurement of cortisol and corticosterone, using CBG we have used plasma treated with charcoal in order to obtain our cortisol-free CBG to use for the assay. Whenever plasma treated in this way has been used, the blanks have always tended to be negative and very high. On the other hand, if one obtains the binding protein, CBG from a patient who has been totally adrenalectomized; and thus avoiding the charcoal step, then the blank becomes very much better and has never been proven to be negative. Also in the aldosterone assay, where one uses an antiserum and there is no charcoal step, the blank has so far never been negative, it has always been positive. There is, I think, a possibility that the negative blank may arise because of the charcoal treatment of the binding protein.

Pizarro: In our case, the negative blank was not a function of the previous treatment of the plasma that served as a source of CBG, because for progesterone, for example, the plasma is just diluted in water. Yet in some cases, there is a negative blank; in 95% of the cases, there is none. Merely changing to a different plasma sample completely eliminates the problem of negative blank, and for progesterone assay, that is the way that was chosen in the remaining 5% of the cases.