real differences were noted in the spectral properties, thermal transition profiles, base composition, or sedimentation behavior of DNA exposed to FQI and DNA isolated from control experiments. These data indicate that, within the limits of the techniques employed in this work, FQI had neither combined with nor altered DNA. It is improbable that a modified nucleic acid was lost in the process of purification, since recoveries in these experiments of both FQI-treated and control nucleic acids as judged from ultraviolet absorption and phosphorus analysis, were uniformly high (88–94 per cent).

The increase in reactivity toward some reagents, which accompanies the denaturation of DNA by heat,[3, 7] prompted experiments with DNA which had been denatured by heating in solutions of low salt concentration.[2] The pH was raised from 7 to 8 in these experiments, a procedure which was found to increase the combination of quinone imides with protein.[1] DNA isolated from such experiments exhibited no changes in either spectral properties or base composition due to exposure to FQI (Table 1).

Experiments with soluble RNA permitted higher concentrations of nucleic acids to be used and offered additional possibilities for reactive sites. Soluble RNA isolated after exposure to FQI at concentrations of nucleic acid four times greater than those used in the DNA experiments was also unchanged with respect to spectral properties, base composition or thermal transition behavior when compared with control preparations (Table 1).

The experiments reported here have provided no evidence that the *o*-quinone imide FQI combines with or alters nucleic acids. The fact that the methods used in this study have detected the combination of other fluorene compounds with nucleic acids[3, 8] strengthens the conclusion that, in contrast to its ability to react with protein,[1] FQI does not react with or modify DNA or RNA. The reactivity of FQI is, therefore, similar to that of the related *o*-quinone imine,[2] but differs from the reactivities of *N*-2-fluorenylhydroxylamine[8, 9] and *N*-acetoxy-2-acetylaminofluorene,[3, 10] which react with both protein and nucleic acid. The N atom of N-acetoxy-2-acetylaminofluorene has been shown to react with the 8-carbon of guanine[11] and a similar mechanism has been proposed for reaction of N-2-fluorenylhydroxylamine with nucleic acids.[8] Combination of *o*-quinone imides[1] and *o*-quinone imines[6] with nucleophiles, however, is believed to result from activation of the fluorene nucleus. These considerations prompt the suggestion that combination of derivatives of the carcinogen 2-acetylaminofluorene with nucleic acid may be favored by activation of the N atom rather than of the aromatic ring.

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Effects of triamcinolone on isolated rat-liver mitochondria

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ON REVIEWING effects and actions of steroid hormones, triamcinolone (a synthetic glucocorticoid) seems peculiar in two respects. First, when used for a long term treatment of rheumatoid arthritis or

bronchial asthma, triamcinolone develops at times singular side effects which include, among others, body weight reduction, muscular atrophy and electrolyte imbalance.[1] Second, triamcinolone is the only steroid hormone which was reported at a reasonable concentration to uncouple oxidative phosphorylation of mitochondria *in vitro*.[2-7] The reported triamcinolone action in mitochondria appears similar to that of valinomycin[8, 9] and parathyroid hormone[10] with regard to the K^+ requirement. This study was designed originally in an attempt to characterize the triamcinolone action in mitochondria by comparing it with that of valinomycin and parathyroid hormone, however, an unexpected conclusion was arrived at, i.e. "triamcinolone effects" in mitochondria was possibly due to contaminant(s) of the sample.

Isolation of the rat-liver mitochondria and measurements of the mitochondrial functions were performed as were described in the preceding papers.[9-11] The terms coined by Chance and Williams[12] were adopted to describe metabolic states of mitochondria, and the P/O, Ca/O and Ca/ADP ratios were calculated according to Chance and Williams[12] and Chance.[13] The mitochondria used in this study responded in the control experiments with the ratios satisfactorily close to the theoretical values; for example, 1·86, 3·70 and 1·99 were obtained for P/O, Ca/O and Ca/ADP ratios respectively when succinate was employed as a substrate. The details of the experiments were also explained in the legends of the figures. Triamcinolone samples used throughout this study were generous gifts from Lederle (Japan) Co., Ltd. and Japan Squibb Co., Ltd. Purity of the samples was examined by measuring melting points and absorption spectra and compared with the authentic samples. In this report are mentioned the results obtained with a batch of about 96·6% pure triamcinolone as calculated from optical density measurements at 238 mμ in ethanol and the results obtained with another batch of optically 100% pure triamcinolone. The samples, the purity of which entered between 95 and 98%, induced similar responses in mitochondria. All samples had the same melting point (262°), no significant amount of residue after ashing (<0·1%) and no appreciable heavy metal contaminant (<0·003%). Because a possibility still exists that the optically pure stuff contains contaminant(s) physico-chemical characteristics of which are similar to that of triamcinolone, these two batches are called in this paper arbitrarily as "optically pure" and "optically impure" samples. All were parts of the stuff commercially distributed as "triamcinolone" and widely consumed in Japan as an anti-inflammatory steroid.

Although most of the results reported by Gomez-Puyou and others[2-7] could be reproduced with the optically impure triamcinolone sample, strikingly different results were obtained with the optically pure stuff. When added to the isolated mitochondria, the former material produced a release of the state 4 respiration (Fig. 1) at apparent concentrations of triamcinolone down to 1×10^{-6} M. The latter, on the other hand, was quite inert up to the apparent concentration of 1×10^{-4} M, insomuch as the state 4 respiration, the state 3 respiration induced either with ADP or 2,4-dinitrophenol, respiratory control ratio, P/O ratio and Ca/O ratio were concerned. This general statement held applicable both in the presence and absence of K^+, Mg^{2+} or permeant anions (acetate and phosphate), and whether succinate and/or the other substrates of the NAD dependent dehydrogenases in mitochondria were employed as an electron donor. Because both samples had almost the same biological potency as judged from the clinical studies, it is almost certain that the specimens contained similar amount of a glucocorticoid, triamcinolone in this case, and spectrophotometric studies and melting point determinations offered another evidence implicating the main constituent of the specimens being triamcinolone. The discrepancy in their effects in *in vitro* system, therefore, derives probably from a contaminant(s) of the samples. One possibility is that the contaminant(s) in the optically pure sample inhibited the triamcinolone action in mitochondria. This was ruled out by the fact that the intact mitochondria as well as mitochondria pretreated with the optically pure stuff reacted equally well to the subsequent addition of the optically impure triamcinolone sample (Fig. 1). The remaining possibility is that the contaminant(s) in the optically impure sample was the real agent which produced such *in vitro* effects as have been attributed to triamcinolone itself. If this were the case, the contaminant(s) should be of quite a high biological potency. The triamcinolone sample at concentration of 0·4 μg/ml, therefore the contaminant(s) at concentration of less than 0·014 μg/ml, induced a significant stimulation of the mitochondrial respiration *in vitro*.

This agent, demanding K^+ for the uncoupling effect (Fig. 2 and references 5 and 7) may differ from 2,4-dinitrophenol, dicoumarol and Ca that develops the full uncoupling effect in the absence of K^+. Along with K^+ the triamcinolone contaminant(s) required permeant anions such as phosphate and acetate for the maximal stimulation of the mitochondrial respiration (Fig. 2). These results, simulating

the events in the respiratory bursts induced by valinomycin[9] or parathyroid hormone,[10] suggest an induction of energy dependent K^+ uptake in mitochondria as a cause of the respiratory stimulation (see refs 9 and 10 for this reasoning). The K^+ effect in the "triamcinolone" pretreated mitochondria was neither reproduced nor influenced by the addition of Mg^{2+}. Thus the mode of action of the contaminant(s) is definitely different from that of parathyroid hormone but rather similar to that of valinomycin.

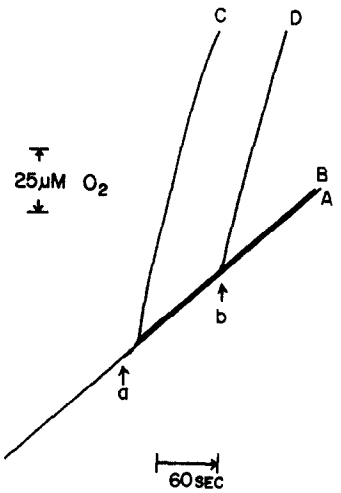

FIG. 1. Effect of triamcinolone on the respiration of the isolated rat-liver mitochondria.

Oxygen concentration in the 3 ml reaction medium that consisted of mannitol 225 mM, sucrose 75 mM, Tris succinate 5 mM, Tris glutamate 5 mM, K Cl 20 mM, Mg Cl_2 5 mM, Tris phosphate 10 mM (Ph 7·4) and mitochondria (about 3 mg protein/ml) was measured by a Clark type oxygen electrode and recorded as a function of time which proceeds from the left to the right in this figure. Triamcinolone was dispersed in propylene glycol to make a 5×10^{-2} M solution. At the point indicated by the arrow *a* 20 μl of the dispersion of the optically pure triamcinolone (B,D) or that of the optically impure triamcinolone (C) was added, and at the point indicated by the arrow *b* 20 μl of the dispersion of the optically impure triamcinolone was added (D). This amount of the vehicle, i.e. propylene glycol, caused a slight increase in the respiration, the course of which was similar to the case of trace B. None was added in trace A. A significant burst of respiration was observed with the solution of the optically impure triamcinolone at the final triamcinolone concentration of 1×10^{-6} M.

Note that the optically pure triamcinolone neither caused a release of respiration nor influenced the course of respiratory stimulation induced by the subsequent addition of the optically impure triamcinolone sample.

However, any statement on the action of the contaminant(s) seems premature until experiments are carried out with an isolated bioactive material. *In vivo* effects of the agent have also to be clarified, because the clinically observed peculiar side effects, for which triamcinolone has been blamed might be the results of the action of the contaminant(s). Further studies on the triamcinolone contaminant(s) await isolation and identification of the active material, efforts for which are in progress in our laboratory.

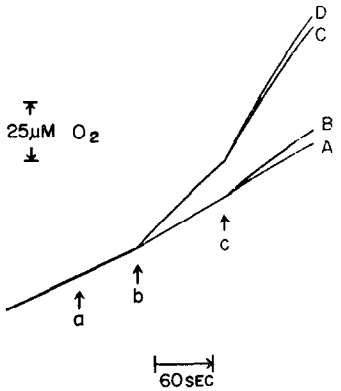

FIG. 2. Effect of ions on "triamcinolone" induced respiratory stimulation in mitochondria.

Rat liver mitochondria (about 1 mg protein/ml medium) were incubated in a 3 ml medium that was composed of mannitol 225 mM, sucrose 75 mM, Tris succinate 5 mM and Tris glutamate 5 mM, Ph 7·4. Oxygen concentration of the medium was recorded with the same notations as in Fig. 1.

Either 5 μl of 50 mM suspension of the optically impure triamcinolone sample (C,D) or 5 μl of the vehicle (propylene glycol: A, B) was added at the time pointed by the arrow *a*. At the time *b*, 5 μl of 2 M K Cl was added in all cases. At the time *c*, 10 μl of 1 M Tris phosphate (Ph 7·4) was added in experiments B and D, and 50 μl of 1 M Tris acetate (Ph 7·4) in experiments A and C. Nearly the same results were obtained when the foregoing medium was fortified with 7 mM (final concentration) Mg Cl_2.

Note that both K^+ and permeant anion (phosphate or acetate) were necessary for the maximal stimulation of mitochondrial respiration by the triamcinolone contaminant(s).

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