titrated at long intervals. 3 M.H.D. of hemolysin and 3 per cent. suspension of packed red cells in saline are employed.

Hyperimmune sera prepared with homologous infected brain tissue have been tested. In contrast to guinea pig sera, rabbit sera usually and mouse sera often give non-specific reactions with unrelated antigens or with normal brain. This effect has been thought to be due to thermolabile substances (Takenomata; Mackie and Finkelstein[2]) and has been removed largely in our tests by establishing temperatures of inactivation for guinea pig serum of 56°, mouse serum, 60°, rabbit serum, 65°. All sera are heated for 20 minutes. This procedure eliminates non-specific as well as anti-complementary reactions without materially disturbing the specific effect (Table I).

TABLE II

COMPLEMENT-FIXATION TESTS WITH MOUSE BRAIN ANTIGENS AND MOUSE IMMUNE SERA

Antigens	Sera inactivated for 20 minutes at 60° C.									
	St. Louis No. 3	Japanese B No. 2604	Japanese B No. 17	Japanese B No. 12	Lymphocytic choriomeningitis	Eastern equine encephalomyelitis	Louping-ill	Rabies (fixed)	Rabies (street)	Saline
St. Louis No. 3	*1/32	0	0	0	0	0	0	0	0	0
Japanese B No. 2604	0	1/32	1/32	1/64	0	0	0	0	0	0
" " No. 17	0	1/32	1/64	1/128	0	0	0	0	0	0
" " No. 12	0	1/32	1/128	1/128	0	0	0	0	0	0
Lymphocytic choriomeningitis	0	0	0	0	1/128	0	0	0	0	0
Eastern equine encephalomyelitis	0	0	0	0	0	1/64	0	0	0	0
Louping-ill	0	0	0	0	0	0	1/64	0	0	0
Rabies (fixed)	0	0	0	0	0	0	0	1/8	1/16	0
" (street)	0	0	0	0	0	0	0	1/8	1/32	0
Saline	0	0	0	0	0	0	0	0	0	–

* 1/32 = Highest dilution at which serum gave a 2+ or better reaction.
0 = No reaction in any of the tubes, the first dilution being usually 1/3 or 1/4.
– = Not tested.

The specific reaction is carried out with 0.25 cc of undiluted antigen, plus two full units of complement in 0.5 cc volume, and 0.25 cc of serum. The serum is used in twofold dilutions commencing with 1 to 3 or 1 to 4. These reagents are placed in the icebox 18 hours and then left at room temperature for one half hour. The hemolytic system is then added, consisting of 0.25 cc of the 3 per cent. suspension of sheep cells plus 0.25 cc of hemolysin containing 3 M.H.D. The total volume per tube is then 1.5 cc. The tubes are incubated at 37° C. for one half hour. The degree of hemolysis resulting in each tube is expressed from 0, indicating complete hemolysis, to 4, indicating no hemolysis.

Specific complement-fixation has been obtained with the viruses of St. Louis encephalitis, Japanese B encephalitis, Eastern equine encephalomyelitis, lymphocytic choriomeningitis, louping-ill and rabies (fixed and street). Table II shows the type of result obtained.

Sera have been shown to react in dilutions as high as 1 to 192 and antigens 1 to 128. Rabies antigen is destroyed at 70° C. in 30 minutes. It is affected but little by ultraviolet light irradiation sufficient to render the preparation avirulent. Centrifugation in the high-speed vacuum centrifuge or filtration through Berkefeld N candles, though reducing the virulence considerably (10,000 to 100,000 times), does not alter the antigenicity materially.

J. CASALS
R. PALACIOS

THE ROCKEFELLER INSTITUTE
FOR MEDICAL RESEARCH,
NEW YORK, N. Y.

[2] N. Takenomata, *Zeitschr. Immunitätsforsch*, 41: 508, 1924; T. J. Mackie and M. H. Finkelstein, *Jour. Hyg.*, 28: 172, 1928–29.

ALCOHOLIC AND NON-ALCOHOLIC KETOSTEROIDS AND THE ZIMMERMAN COLOR REACTION[1]

IN the course of isolating steroids from ether extracts of acid-hydrolized urines from cancerous and non-cancerous persons we noticed that the non-alcoholic ketonic fraction of the neutral material contributed considerably to the total 17-ketosteroid titer as determined by the Zimmerman[2] reaction. Since androsterone, a 17-keto hydroxy steroid, is chiefly responsible for the androgenic activity of urinary extracts, this observation may partially explain the divergence existing between the relatively high 17-ketosteroid colorimetric titer and the biological activity.

Evidence has recently been obtained that the non-alcoholic ketonic fraction contains steroid compounds. Burrows *et al.*[3] obtained $\Delta^{3:5}$ androstadiene-17-one

[1] Aided by grants from the Dazian Foundation for Medical Research and the National Research Council Committee for Problems of Sex. Works Progress Administration Project No. 65–1–14–2949.

[2] W. Zimmerman, *Ztschr. Physiol. Chem.*, 233: 257, 1935.

from the urine of a man with a malignant tumor of the adrenal cortex. Engel[4] isolated two crystalline compounds from the non-alcoholic ketonic fraction of normal male urine. Hirschmann[5] obtained androstenone-17 from the urine of ovariectomized women. We have obtained similar crystallisates from various human urines.[6]

The following table gives the colorimetric titers of (1) the total neutral, (2) the ketonic neutral, (3) the alcoholic ketonic neutral and (4) the non-alcoholic ketonic neutral fractions of a set of pooled urines from non-cancerous and cancerous men and women. The urines were hydrolyzed by boiling for seven minutes after adding concentrated HCl to 15 per cent. by volume. The ketonic material was separated into alcohols and non-alcohols by half-esterification with succinic anhydride.

TABLE 1

THE COLORIMETRIC TITER OF URINARY KETOSTEROIDS IN VARIOUS FRACTIONS OF POOLED HUMAN URINES. ALL VALUES IN MGM EQUIVALENT OF 17-KETOSTEROID PER LITER

Source of urine	Total neutral fraction	Total ketonic fraction	Alcoholic ketonic fraction	Non-alcoholic ketonic fraction
Non-cancerous males .... (188 liters)	14.70	11.62	5.73	5.51
Cancerous males .... (476 liters)	3.90	3.68	1.65	1.73
Non-cancerous females ... (146 liters)	5.69	5.38	2.66	2.00
Cancerous females ... (231 liters)	3.99	3.58	1.74	1.52

It can be seen that roughly half of the titer of the ketones lies in the non-alcoholic fractions. It is interesting that these data on pooled urines confirm previous findings on individual specimens that cancerous persons of both sexes excrete approximately the same amounts of neutral ketosteroids, whereas in the non-cancerous persons there is a clear sex difference and a higher output than in cancerous persons.[7]

If the non-alcoholic ketosteroids are not excreted as conjugated compounds (it is difficult to see how they can be conjugated), then they should be found in full quantity in unhydrolyzed urine. Accordingly, we made a thorough ether extraction of freshly voided male urines (collected from 9 males and extracted within two hours of voiding). This produced 0.3 mgm 17-ketosteroid equivalent per liter of non-alcoholic ketone; the residue after acid hydrolysis yielded an additional 3.2 mgm per liter and 5.1 mgm per liter of alcoholic ketosteroid. We satisfied ourselves by a number of recovery experiments that the separation by succinic anhydride can be successfully carried out on this micro-scale. As little as 50 micrograms of dehydro-androsterone may be isolated in 90 per cent. yield in the alcoholic fraction. The indications, therefore, are that the bulk of the non-alcoholic steroid material arises as a result of the hydrolysis employed.

Since the foregoing findings cast doubt on the probability that the non-alcoholic ketones are true excretion products we have conducted experiments on the effects of acid hydrolysis on androsterone and dehydroandrosterone. Preliminary data indicate that a considerable quantity of each is converted into non-alcoholic material which gives the Zimmermann color reaction. Butenandt and Dannenbaum[8] obtained a chloroketone from urine which they consider a product of HCl hydrolysis. It is notable too that all the non-alcoholic urinary ketones thus far identified might conceivably arise by dehydration of the hydroxyketonic material in the course of acid hydrolysis.

The detailed data of this work and related investigation will be reported elsewhere.

GREGORY PINCUS
WILLIAM H. PEARLMAN
CLARK UNIVERSITY

p-AMINOBENZOIC ACID, A VITAMIN

ABOUT a year ago, Woods and Fildes[1] reported the anti-sulfanilamide activity in vitro of p-aminobenzoic acid. In April, Woods[2] found that yeast extracts contain a substance which nullifies the inhibitory action of sulfanilamide on the growth of hemolytic streptococci, and presented circumstantial evidence that the yeast factor may be p-aminobenzoic acid. In December, Rubbo and Gillespie[3] recovered p-aminobenzoic acid as the benzoyl derivative from yeast and concluded it to be a bacterial growth factor. Experiments conducted in this institute indicate that p-aminobenzoic acid, considered to be an essential metabolite for bacteria by Fildes,[4] is a vitamin, namely, a chromotrichia factor for the rat and a growth-promoting factor for the chick.

One hundred black or piebald rats were placed at weaning age on the basal ration GH–1, consisting of Cerelose 70 per cent., casein 18 per cent., salts 4 per cent., agar 2 per cent., soybean oil 2 per cent., Crisco 2 per cent., and cod liver oil 2 per cent., and received

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